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ESPECTROS DE TAMANHOS DO ZOOPLÂNCTON, PARTÍCULAS EM SUSPENSÃO E MICROPLÁSTICOS EM AMBIENTES ESTUARINO E COSTEIROS DO ATLÂNTICO TROPICAL

Recife
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Tese apresentada ao Programa de Pós-Graduação em Oceanografia da Universidade Federal de Pernambuco (PPGO-UFPE), como requisito parcial para a obtenção do título de Doutor em Oceanografia.
Área de concentração: Oceanografia Biológica

Orientador: Prof. Dr. Ralf Schwamborn.
Orientadora: Profª. Drª. Catarina da Rocha Marcolin

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RESUMO

O conhecimento sobre as partículas em suspensão (séston) é essencial para a compreensão dos ecossistemas estuarinos e marinhos. Esta tese tem como objetivos avaliar as contribuições do mesozooplâncton, de microplásticos e das partículas em suspensão na coluna d'água em amostras de plâncton, e de descrever os espectros de tamanhos destas partículas e organismos. Além disso, foi proposto um novo índice (RMC: Concentração Relativa de Microplásticos) para avaliar o impacto dos microplásticos nas teias tróficas pelágicas. As amostras foram obtidas no estuário do Rio Formoso (área de manguezais), na Baía de Tamandaré (baía aberta, rodeada de recifes tropicais) e na plataforma continental (da linha dos recifes costeiros até a isóbata de 30 m) ao largo de Tamandaré (Pernambuco, Brasil). Arrastos subsuperficiais (profundidade amostral: 0-0.6 m) horizontais foram realizados durante dois anos (abril / 2013 a maio / 2015) em intervalos bimestrais com redes de plâncton (malhas: 200 e 300 μm) durante as estações seca e chuvosa. E em seguida as amostras coletadas foram fixadas em formol (concentração final de 4%) e tamponadas com tetraborato de sódio (5 g L^{-1}). As amostras foram analisadas através da obtenção do peso úmido (biomassa sestônica), análise por imagem (ZooScan) e espectroscopia por infravermelho (FTIR). As análises das tipologias das partículas mostrou que a composição das partículas biogênicas (detritos vegetais, macroalgas, agregados marinhos e exúvias) seguiu o padrão esperado, com mais matéria vegetal (detritos de manguezal) no estuário rodeado de florestas de manguezais. As concentrações mais elevadas de microplásticos totais (Polipropileno + Polietileno + Nylon), PP (Polipropileno) e PE (Polietileno) também foram observadas no ambiente estuarino, indicando um gradiente decrescente para o oceano a partir de fontes terrestres. A RMC indicou que a Baía representa o ecossistema mais impactado (RMC: 2,4% no estuário, 5,1% na Baía e 2,0% na plataforma), para microplásticos totais e PP & PE. Já a plataforma continental foi mais severamente impactada (concentração relativa mais alta) com fibras de nylon. A análise dos espectros de tamanhos mostrou que os organismos planctônicos, microplásticos e outras partículas seguem um padrão semelhante em seus espectros de tamanhos, com maiores concentrações em faixas de tamanhos menores e um declínio log-linear da concentração com o volume ou tamanho das partículas. Essa abordagem inovadora abre novas perspectivas para o estudo dos ambientes pelágicos estuarinos e marinhos tropicais.

Palavras-chave: peso úmido; Zooscan, FTIR; meso-seston; polipropileno; polietileno; nylon; concentração relativa de microplásticos.

ABSTRACT

Knowledge about suspended particles (seston) is essential for understanding estuarine and marine ecosystems. The objective of this thesis is to evaluate the contributions of mesozooplankton, microplastics and particles suspended in the water column in plankton samples, and to describe the size spectra of these particles and organisms. In addition, a new index (RMC: Relative Concetration of Microplastics) has been proposed to assess the impact of microplastics on pelagic food webs. Samples were taken from the Rio Formoso estuary (mangrove area), Tamandaré Bay (open bay, surrounded by tropical reefs) and the continental shelf (from the coastal reef line to the 30 m isobath) off Tamandaré (Pernambuco, Brazil). Horizontal subsurface trawls (sample depth: 0-0.6 m) were carried out for two years (April / 2013 to May / 2015) at bimonthly intervals with plankton nets (meshes: 200 and 300 μm) during the dry and wet seasons. The collected samples were fixed in formalin (final concentration of 4%) and buffered with sodium tetraborate (5 g L^{-1}). The samples were analyzed by obtaining wet weight (sestonic biomass), image analysis (ZooScan) and infrared spectroscopy (FTIR). The analysis of particle typologies showed that the composition of biogenic particles (plant derived, macroalgae, marine aggregates and exuvia) followed the expected pattern, with more vegetable matter (mangrove debris) in the estuary surrounded by mangrove forests. The highest concentrations of total microplastics (Polypropylene + Polyethylene + Nylon), PP (Polypropylene) and PE (Polyethylene) were also observed in the estuarine environment, indicating a decreasing gradient to the ocean from terrestrial sources. The RMC (Relative Concentration of Microplastics) indicated that the Bay represents the most impacted ecosystem (RMC: 2.4% in the estuary, 5.1% in the Bay and 2.0% on the shelf), for total microplastics and PP & PE. The continental shelf was more severely impacted (higher relative concentration) with nylon fibers. Analysis of the size spectra showed that planktonic organisms, microplastic, and other particles follow a similar pattern in their size spectra, with higher concentrations in smaller size ranges and a log-linear decline in concentration with particle volume or size. This innovative approach opens up new perspectives for the study of estuarine and marine pelagic tropical environments.

Keywords: wet weight; Zooscan; FTIR; meso-seston; polypropylene; polyethylene; nylon; relative concentration of microplastics.

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1 INTRODUÇÃO GERAL

O séston é composto por basicamente dois grandes grupos: I. organismos do plâncton e II. detritos, sejam eles orgânicos ou inorgânicos (Blanchot et al., 1989; Odum, 2004; Frigstad et al., 2011; Gladstone-Gallaghe et al., 2016, Lins-Silva et al., 2019; 2021). Os componentes bióticos do séston, tais como os agregados e neve marinha, por exemplo, são extremamente importantes nos ecossistemas pelágicos marinhos uma vez que desempenham papel fundamental na transferência de energia trófica pelágica e também por participarem dos processos biogeoquímicos, como a ciclagem de nutrientes para as camadas mais profundas do oceano através da bomba biológica de carbono (Schumann e Rentsch, 1998; Schwamborn et al., 2002; Schwamborn et al., 2006; Stemmann e Boss 2011; Ohman et al., 2012).

Apesar do conhecimento sobre a importância das partículas no plâncton, a maior parte dos estudos oceanográficos focaram apenas no zooplâncton, (Schwamborn et al., 1999; Boltovskoy, 1999; Melo Júnior et al., 2007; Santos et al., 2009), muitas vezes, negligenciando o papel das partículas suspensas no abastecimento das cadeias alimentares e ciclos biogeoquímicos (Young et al., 1996; Schumann e Rentsch, 1998; Schwamborn et al., 2002, 2006; 2008; Checkley et al., 2008). Entretanto, estudos recentes mostraram que partículas não vivas (por exemplo, detritos vegetais e agregados marinhos) ocupam parte significativa do séston e constituem uma porção significativa das amostras comuns de plâncton, superestimando os cálculos de biomassa úmida nos ambientes marinhos, principalmente em ambientes costeiros tropicais (Lins-Silva et al., 2019; 2021). Além disso, os ecossistemas costeiros também apresentam altas concentrações de partículas antropogênicas, como os microplásticos (Chong et al., 2001; Barnes et al., 2009; Lins-Silva et al., 2021).

Os microplásticos, minúsculos grânulos de plástico derivados da degradação de macroplásticos, são fragmentos de plásticos cujo tamanho não excede 5 mm de diâmetro (Derraik, 2002; Thompson et al., 2004; Ryan et al., 2009). A sua presença no oceano aberto foi destacada, pela primeira vez, na década de 1970 (Carpenter e Smith, 1972). Desde então, os estudos mostram que os microplásticos estão cada vez mais presentes e persistentes nos ambientes marinhos em toda a coluna de água, encontrados em suas maiores concentrações ao longo da costa e nos giros meso-oceânicos e, sua presença nos ambientes marinhos têm potencial de causar graves danos à biota (Derraik, 2002; Moore, 2008; Barnes et al., 2009;

Cole et al., 2011), como, por exemplo a obstrução do aparelho digestivo e alterações nas taxas de crescimento e reprodução.

Tanto o zooplâncton e as partículas biogênicas (detritos vegetais, agregados marinhos e macroalgas) e os microplásticos, são tradicionalmente estudados através de amostragem com redes de plâncton de malha fina, contagens, identificação e classificação manuais sob o auxílio de um microscópio (Blanchot et al., 1989; Neumann-Leitão et al., 1998; Browne et al., 2008). Apesar de eficiente, tais metodologias demandam bastante tempo e não permitem diferenciar os componentes do séston e dessa forma quantificar a contribuição real do zooplâncton e das partículas em suspensão na coluna d'água. Dessa forma, métodos alternativos para estudar o plâncton incluindo os sistemas de análises de imagem (Schumann e Rentsch, 1998; Grosjean et al., 2004; Gorsky et al., 2010) estão cada vez mais sendo utilizados.

As metodologias de análises de imagens (*in situ* ou em laboratório), em conjunto com ferramentas de classificação, têm se tornado popular nos últimos 30 anos. O desenvolvimento de dispositivos ópticos de bancada, como, por exemplo, o ZooScan, fornece imagens de boa qualidade do zooplâncton e das partículas suspensas na coluna d'água das amostras de plâncton preservadas (Grosjean et al., 2004; Gorsky et al., 2010), também pode auxiliar nos cálculos de contagem para uma variedade de estudos ecológicos, uma vez que, medidas morfométricas e ópticas são obtidas para cada objeto (vinhetas) escaneado (>300 µm), em um menor tempo de análise (Grosjean et al., 2004; Gorsky et al., 2010; Vandromme et al., 2012; Forest, et al., 2012). No entanto, este dispositivo não é capaz de distinguir a composição química dos objetos escaneados, havendo assim a necessidade de integrar outras ferramentas de análise, como por exemplo a espectroscopia de infravermelho.

A Espectroscopia de Infravermelho com Transformada de Fourier (FTIR) pode ser usada para identificar polímeros e investigar a composição química e intemperismo das partículas, sejam elas naturais (fragmentos vegetais, por exemplo) ou sintéticas (microplásticas) comparando os espectros das amostras com o conjunto de dados conhecidos (Dutra et al., 1995, Barnes et al., 2009; Munajad et al., 2018). No entanto, esta técnica não pode ser aplicada diretamente em amostras de plâncton, já vez que, a água nas amostras absorve luz infravermelha e, portanto, impede esta análise. Dessa forma, protocolos extensivos de preparação de amostras de plâncton foram desenvolvidos, incluindo procedimentos de desidratação lenta e complexa, para permitir uma interpretação confiável dos espectros de FTIR (Dutra et al., 1995, Pinho e Macedo 2005; Munajad et al., 2018).

Assim, os dados de medidas ópticas, obtidos através do ZooScan, e os de espectroscopia, mensurados com o FTIR podem, portanto, fornecer dados das distribuições do zooplâncton, partículas biogênicas e microplásticos com mais precisão e rapidez do que os métodos anteriores.

Para responder às hipóteses ecológicas associadas a eficiência de energia da comunidade planctônica, vários estudos usam a teoria do espectro de tamanho de biomassa normalizada (NBSS - Normalized Biomass Size Spectra; Kerr e Dickie, 2001; Gaedke et al., 2004; Marcolin et al., 2013; Sato et al., 2015). Essa teoria é considerada um método adequado para reunir informações sobre comunidades inteiras de plâncton de forma padronizada (Platt e Denman, 1978; Platt, 1985; Gaedke et al., 2004). Tal teoria associa os interceptos e inclinações de uma regressão linear ajustada ao espectro com taxas de produção de biomassa, eficiência na transferência de energia e interações predadores e presas sugerindo uma relação inversamente proporcional entre o tamanho corpóreo e a abundância, cujos maiores valores de abundância estão representados pelos organismos de menor tamanho (Zhou, 2006; Krupica et al.,2012). O NBSS é calculado usando a fórmula: NBSS = ΔM / Δm, onde ΔM representa o total da biomassa de organismos em um intervalo de tamanhos e Δm representa o tamanho do intervalo, estimado na mesma unidade de peso. As classes de tamanho são criadas considerando um crescimento exponencial, onde o limite superior de cada classe de tamanho é 1.447 vezes maior que o limite inferior, o que fornece um aumento de 1.5 em volume a cada intervalo de tamanho (Krupica et al.,2012; Petrik et al., 2013). Tanto o NBSS, quanto outros índices baseados em tamanho têm sido usados para examinar as diferenças nas comunidades de plâncton (Gaedke et al., 2004), para detectar variações espaciais na biomassa do zooplâncton (Krupica et al.,2012), e para descrever cadeias alimentares estruturadas por tamanho (Marcolin et al., 2013; Sato et al., 2015).

Atualmente, as comparações sobre as mudanças nos espectros de tamanhos de comunidades planctônicas entre diferentes ambientes (costeiros e marinhos) são escassas (Mackas et al., 2012; Macolin et al., 2015). O trabalho apresentado consiste em um dos primeiros estudos a realizar uma análise comparativa na distribuição de tamanhos através de dados de volume do zooplâncton e das partículas em suspensão no gradiente costa – plataforma continental. Estudos sobre a variação da distribuição de tamanhos em regiões costeiras e marinhas são relevantes para a compreensão dos fatores que influenciam a estrutura da comunidade zooplanctônica ao longo do tempo e para tentar predizer o comportamento destes organismos frente às alterações ambientais.

Neste contexto, a avaliação dos espectros de tamanho e de biomassa contribui para entender as mudanças provocadas à dinâmica dos ecossistemas marinhos (Kerr e Dickie, 2001), como por exemplo, a eficiência da transferência de energia entre os ambientes e as interações entre presa/predador, uma vez que, está relacionada com a quantidade de níveis tróficos e a eficiência energética assimilada (ZHOU, 2006). Além de que, este estudo também inclui informações obtidas através das análises de imagens oriundas do equipamento ZooScan, e do espectrofotômetro para distinguir a tipologia e origem (biogênica ou antropogênica), das partículas em suspensão dentre os diferentes ambientes marinhos.

A presente tese é resultado de estudos desenvolvidos no contexto de dois projetos de pesquisa: ST-ESPLAN-Tropic (Séries Temporais de Espectros de Tamanho do Zooplâncton Marinho Tropical, com ênfase nas larvas de Decapoda) cujo objetivo é contribuir para a compreensão dos ecossistemas estuarinos e marinhos tropicais, e quantificar a contribuição dos estuários com manguezal, em termos de organismos e partículas, para o plâncton costeiro através das análises e comparações dos espectros de tamanho do zooplâncton marinho e estuarino tropical em 2 áreas (uma área marinha e uma estuarina) no Nordeste do Brasil. O segundo projeto a que essa tese está vinculada ao Instituto Nacional de Ciência e Tecnologia em Ambientes Marinhos Tropicais, Heterogeneidade Espaço-Temporal e respostas às Mudanças Climáticas (INCT-Amb Tropic), cujo objetivo é avaliar os efeitos da variabilidade climática (sazonal e interanual) sobre os espectros de tamanho, diversidade e estrutura trófica do ambiente pelágico na plataforma continental ao largo do Norte e Nordeste do Brasil. Os resultados apresentados na tese no contexto dos INCT-Amb Tropic estão se referindo à plataforma continental de Tamandaré, Nordeste brasileiro.

2 HIPÓTESES

A tese foi composta baseada nas hipóteses listadas abaixo:

- A estrutura de tamanho das mesopartículas e do zooplâncton é influenciada pela distância da costa, ou seja, existe relação entre a declividade do espectro e a distância da costa;
- Existe maior contribuição de mesopartículas nos ambientes costeiros quando comparados aos ambientes de plataforma continental;
- A composição das mesopartículas e do zooplânton diferencia entre os ambientes.

3 OBJETIVOS

A tese foi conduzida baseada nos seguintes objetivos:

3.1 Objetivo Geral

Descrever os padrões de distribuição de tamanhos e biomassa do zooplâncton e das mesopartículas em suspensão dos ambientes marinhos tropicais da costa do nordeste brasileiro.

3.2 Objetivos Específicos

- Caracterizar, descrever e comparar os espectros de tamanhos e biomassa das mesopartículas e da comunidade zooplanctônica nos diferentes ambientes;

- Descrever as variações espaciais e temporais nos espectros de tamanhos e biomassa das mesopartículas em suspensão na coluna d’água nas áreas costeiras e marinhas tropicais;

- Identificar a tipologia das mesopartículas;

- Avaliar a real contribuição do zooplâncton e das mesopartículas em suspnsão na coluna d’água para a biomassa sestônica entre diferentes ecossistemas**;**

- Avaliar se a tipologia, concentração e volume das partículas diferem entre as áreas costeiras e marinhas tropicais;

3.2.6 Analisar a contaminação por microplásticos e identificar quais ecossistemas são mais impactados por diferentes tipos de microplásticos.

4 ESTRUTURA DA TESE

Esta tese está dividida em três artigos científicos cujos dois dos artigos já foram publicados em periódicos científicos e um será submetido. De modo geral, cada artigo apresenta a relação existente entre as contribuições dos organismos, das partículas em suspensão e dos microplásticos encontrados no plâncton, diferenciados através da análise de imagem e da espectroscopia por infravermelho.

Artigo 1 - *Using image analysis to assess the contributions of plankton and particles to tropical coastal ecosystems*

Estado: Publicado - Estuarine, Coastal and Shelf Science

DOI: https://doi.org/10.1016/j.ecss.2019.02.010

Objetivo geral:

Determinar a contribuição relativa de meso-partículas para a biomassa meso-seston em amostras de plâncton obtidas com redes comuns de plâncton, comparando medidas de peso úmido padrão e análise de imagem.

Hipótese:

Meso-partículas robustas (> 200 µm) constituem um componente relevante para a biomassa sestônica em amostras de plâncton costeira tropical.

Artigo 2 - *A fresh look at microplastics and other particles in the tropical coastal ecosystems of Tamandaré, Brazil*

Estado: Publicado - Marine Environmental Research

DOI: https://doi.org/10.1016/j.marenvres.2021.105327.

Objetivo geral:

Analisar a contaminação por microplásticos através de uma nova abordagem sugerindo um novo índice, RMC (Concentração Relativa de Microplástico), revelando as fontes e sumidouros de partículas biogênicas e antropogênicas. Além disso, discernir os ecossistemas mais impactados por diferentes tipos de microplásticos.

Hipótese:

A tipologia das partículas, concentração e volume (em unidades absolutas e relativas), diferem entre os ecossistemas (estuário de manguezais, baía revestida de recifes de coral e plataforma continental).

Artigo 3 - *What determines the shape of the complete size spectrum (including plankton, microplastics and other particles) in tropical coastal ecosystems?*

Estado: A ser submetido - Progress in Oceanography

Objetivo geral:

Analisar em que circunstâncias a distribuição de zooplâncton (em termos de densidade e volume totais e relativos) e espectros de tamanho são mais impactados por microplásticos e outras partículas suspensas na coluna de água.

Hipótese:

Os espectros de tamanhos do zooplâncton e das partículas diferem entre os ecossistemas (estuário de manguezais, baía beirada por recifes de coral e plataforma continental) e entre os tipos de partículas (1. espectros apenas com zooplâncton, 2. espectros do zooplâncton + partículas biogênicas, e 3. espectros completos, com zooplâncton + todas partículas, incl. microplásticos).

5 ARTIGO 1: USING IMAGE ANALYSIS TO ASSESS THE CONTRIBUTIONS OF PLANKTON AND PARTICLES TO TROPICAL COASTAL ECOSYSTEMS

Publicado - Estuarine, Coastal and Shelf Science

A B S T R A C T

Suspended particulate matter (seston) in aquatic ecosystems contains two compartments: organisms (plankton) and particles. To know the contribution of particles to the pelagic realm is essential to understand the structure and dynamics of aquatic ecosystems. This study aims to determine the relative contribution of particles to the meso-sized seston biomass in plankton samples obtained with common plankton nets (meshes: 200 and 300 µm) comparing wet weight measurements and image analysis using a ZooScan equipment. Samples were obtained in Tamandaré Bay and in the Rio Formoso estuary (Pernambuco, Brazil) during two years (June/2013 to May/ 2015) at bi-monthly intervals during dry and rainy seasons, totalizing 155 samples. The estuarine environment had the highest values of wet meso-seston biomass, abundance, and volume. In the estuary, the relative contribution of particles in volume units (> 55%) was higher than zooplankton. In the bay surrounded by coral reefs, relative particle volume was lower than in the estuary, but still very important (36.86% and 52.15% in the 200 and 300 µm nets, respectively). Zooplankton and particles were more abundant during the dry season. We found significant and positive linear relationships (r2=0.68; $p < 0.0001$), indicating that ZooScan-derived volume data can be confidently used to estimate wet biomass. This study provides a novel approach for the analysis of non-fragile mesoparticles and mesozooplankton in estuarine and marine ecosystems, based on sampling with plankton nets and subsequent analysis with imaging systems. This approach allows new interpretations on the composition of large-sized, robust seston in tropical areas.

Keywords: Wet weight; ZooScan; Volume; Meso-seston.

5.1 Introduction

Suspended particulate matter, *i.e.* seston, can be divided into two basic groups: organisms (plankton) and non-living particles. Biogenic particles, such as aggregates, marine snow (RILEY et al., 2012; PETRIK et al., 2013), and detritus (BLANCHOT et al., 1989; CHONG et al., 2001) are important components of marine pelagic ecosystems because they can be a relevant food source for zooplankton and also play an important role in many processes (SCHUMANN & RENTSCH 1998; SCHWAMBORN et al., 2002; SCHWAMBORN et al., 2006; OHMAN et al., 2012). Although knowing the relative contribution of particles in the seston is key to understand the dynamics of trophic energy flows in marine ecosystems (BLANCHOT et al., 1989; ODUM, 2004; LI et al., 2012; GLADSTONE-GALLAGHER et al., 2016), oceanographic surveys generally focus on the plankton rather than particles. For instance, many studies addressed the zooplankton community structure and plankton wet biomass in tropical coastal environments, especially in estuarine areas (YOUNG et al., 1996; PARK & MARSHALL 2000, SILVA et al., 2004; SCHWAMBORN et al., 2008), but none of them analyzed the contribution of particles. In addition, particles may be fragile, which can cause their disruption in towed plankton nets and, thus, are difficult to quantify in standard formalin-preserved plankton samples.

In spite of several advantages of *in situ* optical devices, such as the VPR, LOPC, and UVP (ASHJIAN et al. 2001, BROUGHTON & LOUGH, 2006; HERMAN & HARVEY 2006; FOREST et al., 2012), it is rarely possible to classify particles using these large, heavy, and cost-intensive devices in standard surveys. Even when such devices can be obtained and effectively deployed, the distinction between zooplankton and particles in a large number of low-resolution and low-quality images due to unexpected orientation, lack of focus or rapid passage in front of the camera hamper a trustworthy classification of many objects with most of these devices. Benchtop imaging devices, such as the ZooScan, do not provide a high spatial resolution since it is necessary to analyze preserved samples in the laboratory. However, these devices provide a much better image quality (perfect focus, perfect flat orientation, no problems with distorted moving images, etc.) that enables an accurate classification and measurement of zooplankton and particle types and a reliable distinction between living (organisms) and non-living matter.

Furthermore, particles from anthropogenic sources such as diverse plastics, toxic and non-toxic paint fragments, asbestos, paper, glass, and wood, among others, arrive in the marine and coastal environments from various sources and are some of the major marine pollutants (COE & ROGERS, 2000). Many studies have been conducted on zooplankton community structure and wet biomass in tropical coastal environments, especially in estuarine areas (YOUNG et al., 1996; PARK & MARSHALL 2000, SILVA et al., 2004; SCHWAMBORN et al., 2008). However, these studies addressed only the total wet biomass, without distinguishing the contribution of particles to seston biomass.

The presence of non-living, robust mesoparticles (> 200 µm) in plankton samples has widely been ignored and most of the current papers dealing with plankton samples still treat particles as noise (e.g., VANDROMME et al., 2012). One of the few studies that attempted to assess the contribution of mesoparticles to the seston in a Malaysian coastal area estimated the difference between total seston biomass and zooplankton biomass (NAKAJIMA et al., 2010). Although Vandromme et al. (2012) differentiated biological and non-biological particles (fibers and detritus) and presented abundance, volume, and their relative contribution to the samples, their approach focused on methodological aspects, such as the quality and speed of imaging analysis. On the other hand, Marcolin et al. (2013) used the ZooScan to separate organisms from particles in plankton samples taken in an offshore shelf area and analyzed the contribution of particles in the shape of the size spectra.

Biomass measurements are necessary steps to quantify the transfer of organic matter and energy flows through plankton food webs (ALCARAZ et al., 2003; LEHETTE & HERNANDÉZ-LEÓN 2009; CRIPPS et al., 2016). There are several methods for the quantification of biomass, but they are not yet well standardized (see PINTO-COELHO, 1972; JACOBS & GRANT, 1978; HARRIS et al, 2000; ALCARAZ et al., 2003). Direct measurements of biomass are often made by an estimate of volume or weight of the samples (PINTO-COELHO, 1972; OMORI & IKEDA, 1984; ALCARAZ et al., 2003; MELO JÚNIOR et al., 2007). Although these are quick, easy, and practical ways to estimate the wet biomass, these methods are unable to identify and classify the different components of the seston (ALCARAZ et al., 2003).

Alternative methods to study plankton include imaging systems (DAVIS et al., 1992; SCHUMANN & RENTSCH 1998; GORSKY et al., 2000; GROSJEAN et al., 2004; GORSKY et al., 2010). These methods are promising for allowing a fast, semi-automatic application in different ecosystems (GORSKY et al., 2000; GORSKY et al., 2010). One of the most commonly used image acquisition equipments is the ZooScan system (GORSKY et al., 2010), which is integrated with the ZooProcess and Plankton Identifier software to allow the generation, analysis, and classification of digital images from preserved plankton samples. This semi-automatic approach does facilitate and assist in the identification and counting, and allows a fast calculation of size, area, and volume for a variety of taxa and ecosystems (GROSJEAN et al., 2004, GORSKY et al., 2010, VANDROMME et al., 2012).

The objective of this study was to determine the relative contribution of particles to the total seston biomass in plankton samples obtained with common plankton nets, comparing standard wet weight measurements and image analysis. We tested the hypothesis that robust mesoparticles constitute a relevant component of the total mass of large-sized, non-fragile seston in tropical coastal ecosystems.

5.2 Methods

5.2.1 Study area

Sampling was conducted in two coastal environments in northeastern Brazil, an estuarine system and an open coastal bay (Fig. 1). This study compares a clear-water coastal environment lined by coral reefs (Tamandaré Bay) with a highly turbid and potentially particle-rich estuarine system lined by mangroves (Rio Formoso estuary).

The Rio Formoso Estuary is located between 8° 39' - 8° 42' S and 35 ° 10' - 35 ° 05' W. This is the second largest estuarine system in the Pernambuco State, comprehending two rivers (Rio Formoso and Rio Ariquindá). It has extensive riverine mangrove forests along the estuarine margins (SILVA et al., 2003). Four mangrove tree species occur in this ecosystem (*Rhizophora mangle* Linnaeus, *Laguncularia racemosa* Gaertn., *Avicennia shaueriana* Staf. and Leechman, and *Conocarpus erectus* Linnaeus.). Muddy sediments are dominant and rich in organic matter, which seems to be the most important source of suspended matter in the estuary (LIRA et al., 1979). Artisanal fisheries, tourism, and the harvesting of crabs and mollusks are the main economic activities in this estuary (SILVA et al., 2003; HONORATO-SILVA, 2009).

Tamandaré Bay (8° 44' - 8° 47' S and 30° 0.5' - 35.07' W) is a shallow coastal embayment that is lined by several parallel sandstone barrier reefs (REBOUÇAS, 1966). Its sediment is classified as carbonate, with high sedimentation rates and low hydrodynamics, because these barriers promote a water imprisonment (REBOUÇAS, 1966; CAMARGO et al, 2007). Macroalgae, soft corals (zoanthids) and scleractinian corals are the most conspicuous primary producers on the surrounding reefs (Santos et al., 2015). Two small creeks, Una and

Mamucabas, discharge into the bay. Tourism and artisanal fisheries are the main economic activities in this region (MOURA & PASSAVANTE, 1993; ARAÚJO & COSTA, 2004).

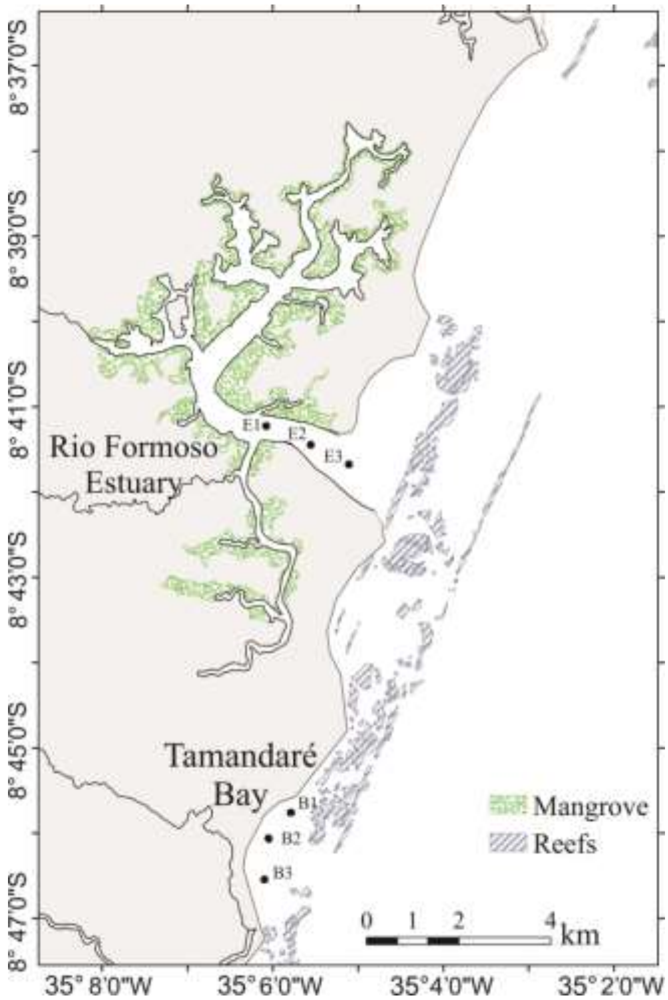

Figure 1. Location of sampling stations in two tropical coastal environments: Rio Formoso Estuary (EST1, EST2, and EST3) and Tamandaré Bay (B1, B2, and B3). Southern Coast of Pernambuco - Brazil. Plankton samples were obtained with conical-cylindrical nets, bimonthly, between June 2013 and May 2015.

Both areas present a warm and humid tropical climate. The annual precipitation is higher between April and August, *i.e.*, the rainy season, while the dry season is from September to March (EMBRAPA - http://www.agencia.cnptia.embrapa.br; NASCIMENTO-VIEIRA, et al., 2010). Both regions are located within a large Marine Protected Area (Federal Decree, October 27, 1997; FERREIRA & MAIDA, 2006). The precipitation is high (average: 2,788.4 mm) and irregular in the coastal region of the Pernambuco State, Brazil, with higher values usually in the rainy season, from March to July (http://www.tamandare.pe.gov.br; APAC – http://www.apac.pe.gov.br).

5.2.2 Plankton sampling

A total of 155 plankton samples were collected in regular bimonthly intervals, during two years, between June 2013 and May 2015. Sampling was conducted during daytime, within two days after the new moon period, at the same tide. During each field campaign, six fixed stations were sampled, three in each area (Figure 1).

Tows were performed using two conical-cylindrical plankton nets, one with a 200-µm-mesh (diameter: 30 cm) and one with a 300-µm-mesh (diameter: 60 cm), by means of simultaneous subsurface horizontal tows during five minutes at a speed of 2 to 3 knots, during ebbing (Rio Formoso Estuary) and flooding tides (Tamandaré Bay). A flowmeter (Hydro-Bios, Kiel) was coupled to the mouth of the nets to estimate the filtered volume. According to Omori & Ikeda (1984), samples were immediately preserved in formaldehyde (4% final concentration), buffered with sodium tetraborate (5 g L^{-1}). Transparency was estimated using a Secchi disk (PREISENDORFER, 1986). *In situ* measurements and vertical profiles of salinity and temperature were obtained using a CastAway (YSI) CTD.

5.2.3 Wet seston biomass

In the laboratory, samples were gently accumulated using an acrylic sieve with a nitex 120-µm-mesh and washed with freshwater to remove the formaldehyde solution prior to determining seston wet weight. Then, interstitial water was gently removed with paper towels placed under the mesh. This procedure took 1 to 15 minutes, depending on the amount of material in each sample. Finally, each sample was weighted on a precision balance (± 0.001 g) to obtain the wet weight (JACOBS & GRANT, 1978; OMORI & IKEDA, 1984; HARRIS et al., 2000). Macrodetritus such as mangrove leaf fragments, macroalgae, and plastics larger than 1 cm were removed from the samples before weighing and weighed separately. Seston biomass was obtained using the formula: $B = WW * V^{-1}$, Where B: total wet seston biomass (mg m^{-3}), WW = wet weight (mg); V: filtered volume (m^3).

5.2.4 Image acquisition and analysis

Plankton samples were digitized using a ZooScan (Hydroptic model ZSCAN03) with a 2400 dpi resolution, following the protocol established by Grosjean et al. (2004; http://www.zooscan.obs-vlfr.fr/). Each sample was diluted in freshwater in a beaker, according to the concentration of organisms, and then gently mixed before the extraction of a 10-ml fraction. The number of objects contained in each scanned fraction ranged from 425 to 5,924 objects.

Images were processed using the ZooProcess software (Version 7.19). The smallest particle size was set to 300 μm of equivalent spherical diameter (ESD). The ZooProcess software isolates images of particles/organisms, *i.e.*, vignettes. For each vignette, several parameters for particle size, gray level, and shape are stored in *.pid files, which were loaded into Plankton Identifier (GORSKY et al. 2010). In the Plankton Identifier (version 1.3.4), we built a training set to provide an algorithm for the automatic classification of vignettes into taxonomic categories. The Random Forest algorithm was chosen following Grosjean et al. (2004). All vignettes were manually validated to correct for misclassification errors. Size parameters were converted from pixels to micrometers, according to the scanner resolution (1 pixel: 10.58 μm).

5.2.5 Zooplankton and particle volume

Biovolume of all zooplankton groups (except fish larvae) was considered to be approximately spherical or ellipsoid. Volume (mm^3) was generally estimated based on the lengths of the major and minor axes of the equivalent ellipse (*i.e.*, an ellipse with the same area and similar height/width ratio as the original vignette). Ellipsoid volume was calculated as: V = $\frac{4}{3}\pi * a^2 * b$, where a = minor axis and b = major axis (VANDROMME et al. 2012; STEMMANN & BOSS 2015). All flat particles and fish larvae were considered as having a flat shape and their volume (mm^3) was calculated based on the surface area (the "area_exc" parameter; mm^2) multiplied by the thickness of each particle type (GROSJEAN et al. 2004). Thickness was measured under a stereomicroscope (Zeiss, Stemi SV6 model) in 30 randomly chosen plankton samples. For each sample, 50 particles were taken from three different categories (opaque, dark, and transparent flat particles), classified according to their shape and grey level. For opaque and dark particles, mean thickness was 781 μm, whereas for transparent flat particles, mean thickness was 319 μm.

5.2.6 Statistical analyses

All data sets were tested for normality and homoscedasticity using Kolmogorov-Smirnov and Levene tests, respectively. Before the tests, $\log_{x+1}$ transformation was applied for the wet biomass data. Since the data distribution was not normal, differences between seasons (dry vs rainy), mesh sizes (200 vs 300 µm), and sampling areas (Rio Formoso Estuary vs Tamandaré Bay) were tested using non-parametric Mann-Whitney tests. Kruskal-Wallis ANOVA was used to test for differences between sampling stations.

Linear regressions were used to investigate the relationships between pairs of variables (zooplankton and particle volume). In the linear regression between total volume and wet sestonic biomass, outliers were removed by means of a residual analysis, using ± two standard deviations as a threshold. All analyses were conducted considering alpha = 0.05 (ZAR, 1996).

5.3 Results

5.3.1 Environmental variables

Both locations (Rio Formoso Estuary and Tamandaré Bay) showed characteristic hydrographic conditions, with much lower salinity and much lower transparency in the estuary in relation to the bay (Fig. 2, Table 1). Euhaline conditions were found in the bay (salinity: 35.00 to 36.54; average: 35.89), while mesohaline to euhaline conditions were observed in the estuary (salinity: 27.03 to 36.43; average: 32.12).

Temperature and salinity varied seasonally in both locations, with a gradual increase through the dry season (September to March). On the other hand, an abrupt drop in salinity was observed in the estuary during the onset of the rainy season in May 2014 (Fig. 2, Table 1).

Estuarine waters were brown and turbid, during all surveys. Secchi depth did not differ significantly between both seasons and the three estuarine stations (average: 1.82 m; range: 1.20 to 3.50 m). In the bay, however, water transparency was significantly higher in the dry season (average: 3.65 m; range: 1.63 to 5.50 m), when low wind intensity and low rainfall led to calm, very transparent waters. In the rainy season, transparency in the bay decreased significantly due to seasonal phytoplankton blooms, which led to a characteristic greenish color in the rainy season (average: 2.63 m; 1.00 to 4.50 m).

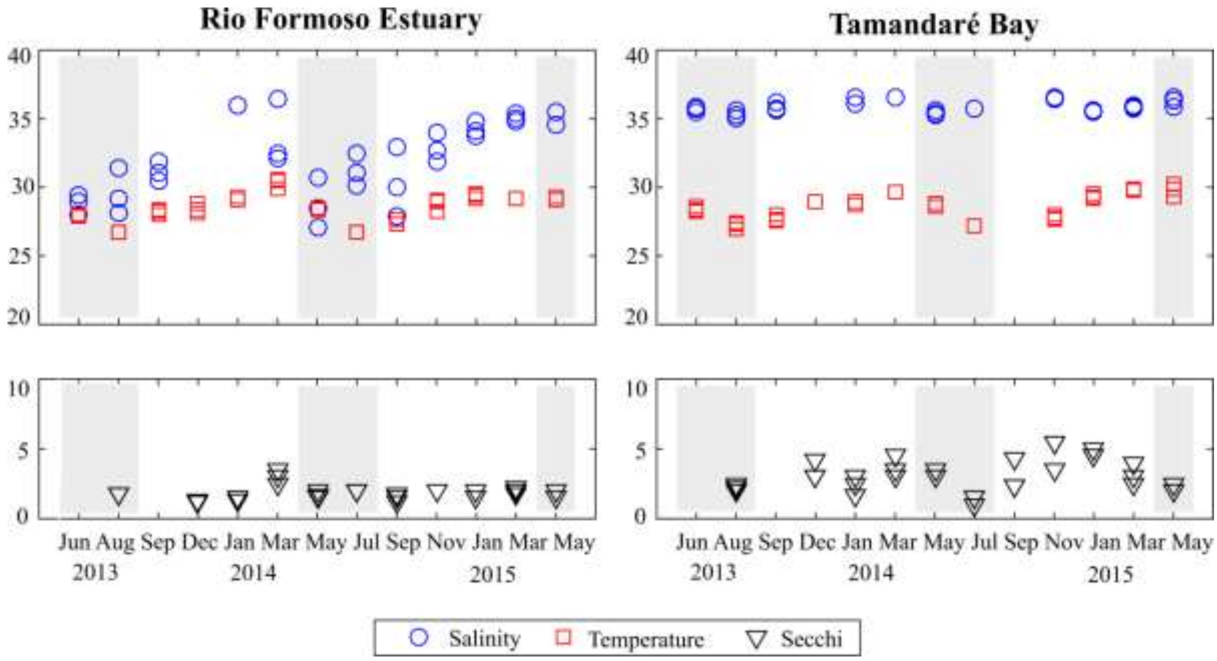

Figure 2. Environmental variables (salinity, temperature, and Secchi depth, in meters). Bimonthly sampling from 2013 to 2015 in the Rio Formoso Estuary and in Tamandaré Bay, Brazil. Shaded areas represent the rainy period.

Table 1. General description of environmental variables. Bimonthly sampling from 2013 to 2015 in the Rio Formoso Estuary and in Tamandaré Bay, Brazil.

	Rio Formoso Estuary				**Tamandaré Bay**			
Variable	Min	Max	Mean ± SD	Median	Min	Max	Mean ± SD	Median
Salinity	27.03	36.43	32.12 ± 2.74	32.15	35.00	36.54	35.89 ± 0.44	35.77
Temperature	26.7	30.54	28.44 ± 1.03	28.44	27.00	30.28	28.66 ±0.95	28.72
Secchi depth	1.20	3.50	1.82 ± 0.50	1.8	1.00	5.50	3.10 ± 1.18	3

5.3.2 Wet seston biomass

Wet seston biomass showed high variability throughout the study period, ranging from 0.5 to 17.95 mg m^{-3} (Table 2). Significantly (p < 0.0001) higher biomasses were observed in the estuary than in the bay. Such a difference between areas was observed for both nets (200 and 300-µm mesh size) and for both seasons (dry and rainy). Biomass was generally higher in the dry season, in both areas. Such significant differences between dry and rainy seasons were found in the estuary, for the 300-µm samples (p = 0.008) and in the bay, for the 200-µm net samples (p = 0.02) (Fig. 3; Table 2).

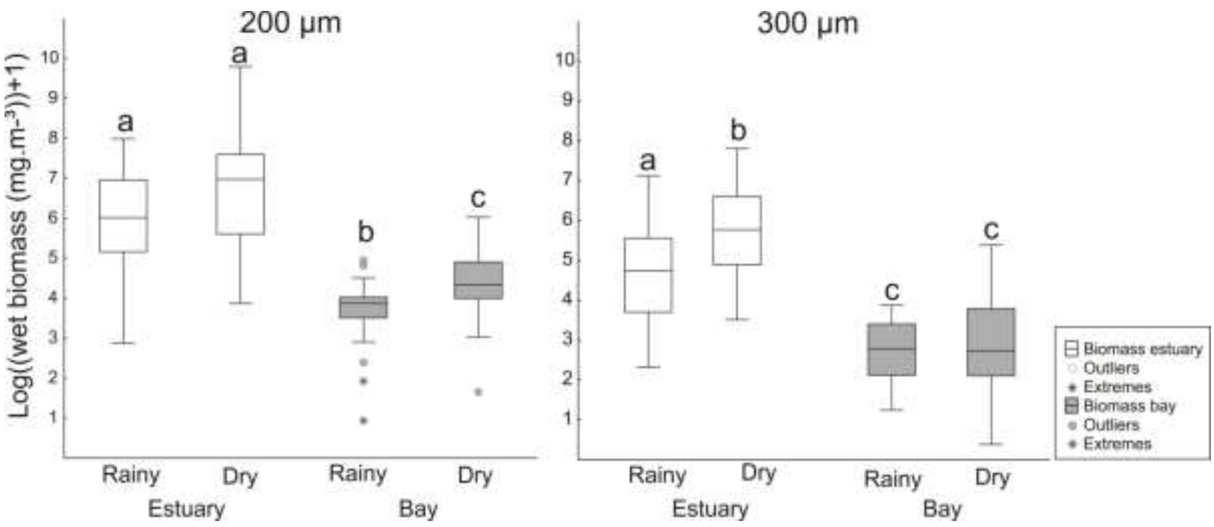

Figure 3. Total sestonic wet biomass (mg m^{-3}) in the different seasons (dry and rainy) and between study areas: Rio Formoso Estuary (white) and Tamandaré Bay (gray), according to the mesh size (200 µm and 300 µm). Bimonthly sampling from 2013 to 2015 in the Rio Formoso Estuary and in Tamandaré Bay, Brazil. n = 155.

5.3.3 Zooplankton and particle abundance

Twenty-six zooplankton taxa were identified in the digital images (Fig. 4). Copepods (Calanoida and Cyclopoida), brachyuran zoea larvae, other decapods, and appendicularians were the most abundant. Particles were classified into 15 categories. The most abundant ones were: opaque, dark, and transparent particles, zooplankton exuviae, and plant fibers.

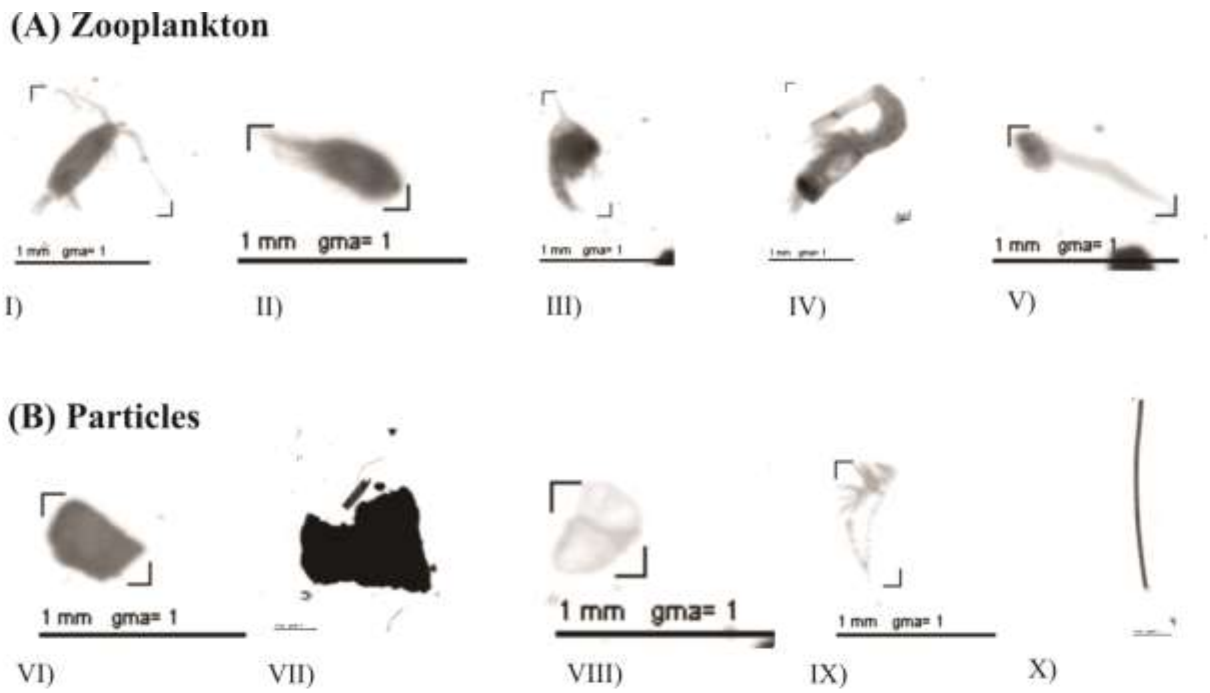

Figure 4. Examples of images (vignettes) obtained with the ZooScan equipment. A) Examples of the most abundant zooplankton groups. I: Calanoida; II: Cyclopoida; III: Brachyuran larvae (zoea); IV: Decapoda (others); V: Appendicularia. B) Examples of the most abundant particles. VI: opaque particles; VII: dark particles; VIII: transparent particles; IX: zooplankton exuviae; X: plant fibers.

Particles were very abundant in both areas, corresponding to 13% of the objects in the Tamandare bay, 69% of the total objects in the estuary; 41% of the objects detected overall. Zooplankton was relatively more abundant in the 300-µm net whereas particles were more abundant in the 200-µm net in both locations (Fig. 5a). However, no significant differences were evident in the zooplankton/particles ratio between areas, since both types of objects were more abundant in the estuary.

The abundances of both zooplankton and particles were highly variable, ranging from 1.6 to 42,536 ind. m^{-3} and from 4.9 to 66,052 objects m^{-3}, respectively (Table 2). Their abundances were generally higher in the 200-µm net, in the dry season, and in the estuary (Table 2). Considering zooplankton, significant differences between seasons were only found in the estuary, for both nets (200-µm: p = 0.006; 300-µm: p = 0.003). Particles were significantly more abundant ($p < 0.0001$) in the estuary than in the bay (Table 2).

Table 2. Wet seston biomass (mg m^{-3}), abundance of zooplankton (ind.m^{-3}) and particles (m^{-3}), and volume of zooplankton and particles (mm³. m^{-3}) according to location, mesh size, and season. Bimonthly samples with two plankton nets (200 µm and 300 µm mesh) from 2013 to 2015 in the Rio Formoso Estuary and in the Tamandaré Bay, Brazil. *n* = 155.

Place	**Rio Formoso estuary**				**Tamandaré Bay**			
Mesh	**200 µm**		**300 µm**		**200 µm**		**300 µm**	
Period	**Rainy**	**Dry**	**Rainy**	**Dry**	**Rainy**	**Dry**	**Rainy**	**Dry**
Parameter								
Wet seston biomass (mg.m^{-3})								
Average	715.9	2,952.4	213.6	585.3	52.7	106.1	18.3	35.6
Minimum	1.7	46.8	9.1	32.4	1.5	4.3	2.4	0.5
Maximum	2,941.9	17,949.1	1,239.6	2,469.5	139.0	413.3	46.2	216.3
Zooplankton abundance (ind. m^{-3})								
Average	2,012.0	9,609.5	677.0	2,350.2	1,284.4	3,863.7	111.5	234.5
Minimum	1.6	303.8	45.4	165.9	35.9	69.5	4.6	8.6
Maximum	15,234.0	42,536.0	3,680.1	6,726.4	7,985.4	15,665.0	490.3	2,182.8
Particle abundance (counts m^{-3})								
Average	7,143.0	20,805.0	1,404.5	4,343.9	290.4	389.3	97.2	69.0
Minimum	45.8	948.0	60.9	83.0	61.6	52.6	4.9	17.5
Maximum	20,241.0	66,052.0	6,055.6	16,156.0	722.3	1,572.2	273.7	232.5
Zooplankton volume (mm³. m-³)								
Average	116.3	464.6	60.9	201.4	44.7	105.9	16.4	28.1

Minimum	0.1	15.1	1.8	13.0	1.3	2.1	1.3	0.6
Maximum	1,145.8	1,688.7	359.2	879.6	193.0	351.4	49.4	248.4
Particle volume (mm³. m-³)								
Average	354.6	932.0	519.8	853.5	21.4	18.7	18.8	87.4
Minimum	1.9	34.7	2.3	3.9	2.7	1.4	0.3	1.7
Maximum	1583.4	2649.7	7644.1	8799.8	137.1	82.6	69.2	1334.7

5.3.4 Zooplankton and particle volume

Particles also showed a high contribution in terms of total volume in the estuary for both mesh sizes, with 44% of all objects in the Bay and 64% of the objects in the estuary corresponding to 54% of the overall counts detected in the ZooScan. Zooplankton, however, had a higher relative volume in the Bay (Fig. 5b).

The volume of both zooplankton and particles were highly variable, ranging from 0.1 to 1,688.70 mm³ m^{-3} and from 0.1 to 8.799,8 mm³ m^{-3}, respectively (Table 2). Highest values were found in the estuary, particularly in the 200-µm-mesh net samples, during the dry season. Significant differences between seasons were found in the estuary, for both nets (200-µm: p = 0.004; 300-µm: p = 0.003), with higher values in the dry season. However, no significant differences were found in the zooplankton: particles ratio between areas and seasons. Significant differences between mesh sizes were found in the bay, with higher total particle volume in the 200-µm mesh (p = 0.03) in the dry season (Fig. 5b).

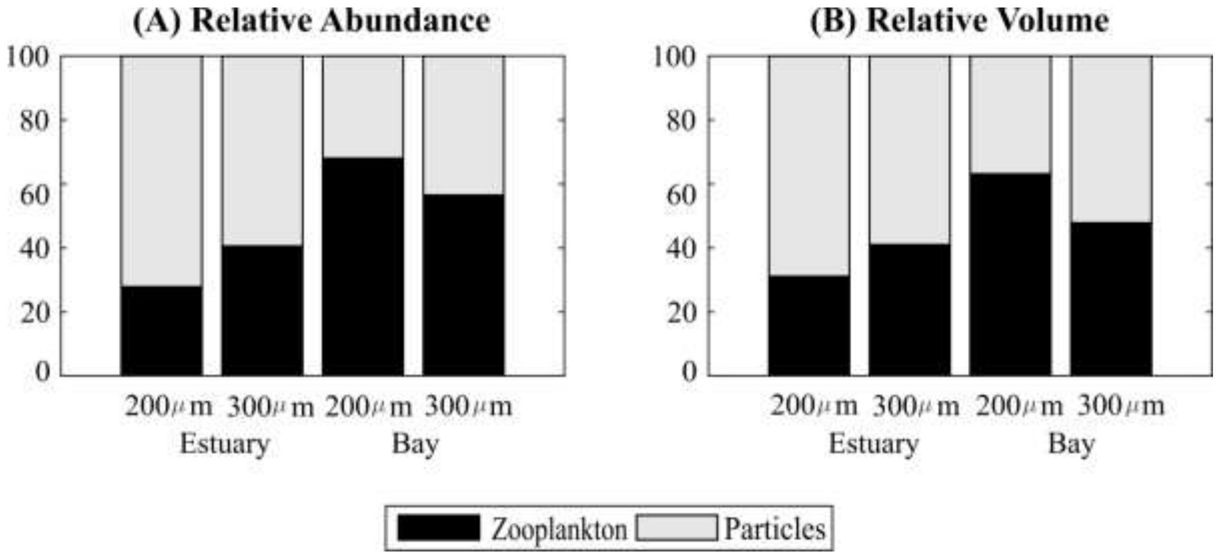

Figure 5. Relative contributions of abundance (A) and volume (B) of zooplankton and particles. Bimonthly samples with two plankton nets (200 µm and 300 µm mesh) from 2013 to 2015 in the Rio Formoso Estuary and in the Tamandaré Bay, Brazil. *n* = 155.

5.3.5 Correlations between zooplankton and particle distributions

In the estuary, significant correlations were found between zooplankton and particles abundance for both the 200-µm (p = 0.002) and the 300-µm nets (p = 0.009). In the Tamandaré Bay, there was also a significant correlation in the 300-µm net (p = 0.03). Considering the volume, significant correlations between zooplankton and particles were found for both nets in the bay (200-µm-mesh: p = 0.02; 300 µm net: $p < 0.0001$; Fig. 6), whereas in the estuary zooplankton and particles were significantly correlated only in the 200-µm samples (p = 0.0005).

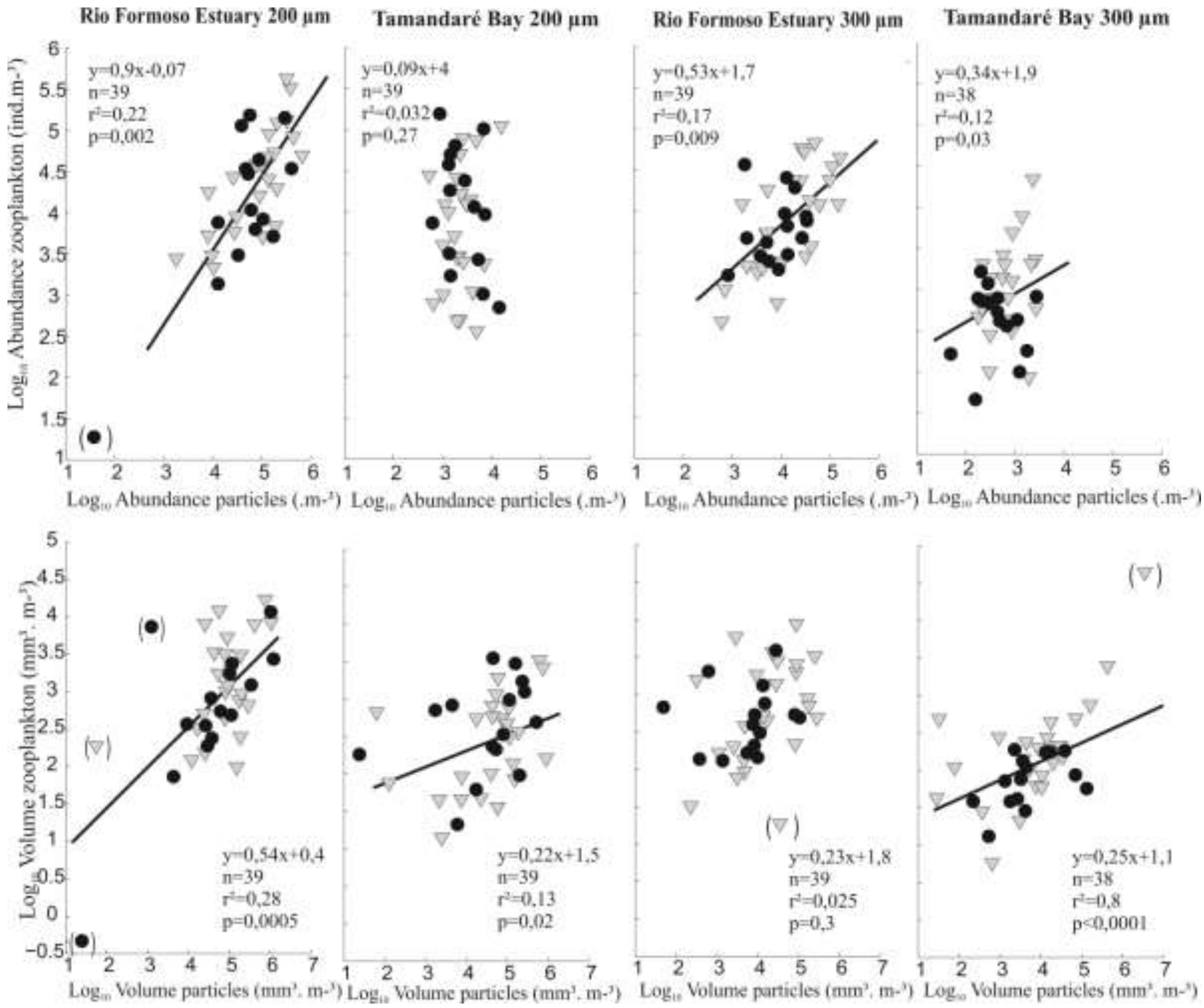

Figure 6. Log_{10} total abundance and total volume of the particles in relation to the total volume of zooplankton. Black circles: rainy season; grey triangles: dry season; brackets: outliers. Bimonthly samples with two plankton nets (200 µm and 300 µm mesh) from 2013 to 2015 in the Rio Formoso Estuary and in the Tamandaré Bay, Brazil. *n* = 155.

5.3.6 Total ZooScan volume vs seston wet biomass

The log-linear model of seston wet mass (Y: $\log_{10}(mg\ m^{-3})$) *vs* ZooScan total volume (X: $\log_{10}(mm^3\ m^{-3})$) showed a highly significant correlation (n = 152; y= 1.006 x - 0.13; r^2= 0.68; $p < 0.0001$; Fig. 7). This indicates that both methods provide similar results, *i.e.*, similar total mass estimates, assuming that the average density of organisms and particles is similar to that of seawater. The slope of the model (1.006 ± 0.05) was not significantly different from 1 (p = 0.91), and the intercept (-0.134 ± 0.121) was not significantly different from zero (p = 0.27), indicating an excellent agreement between both measurements of wet mass.

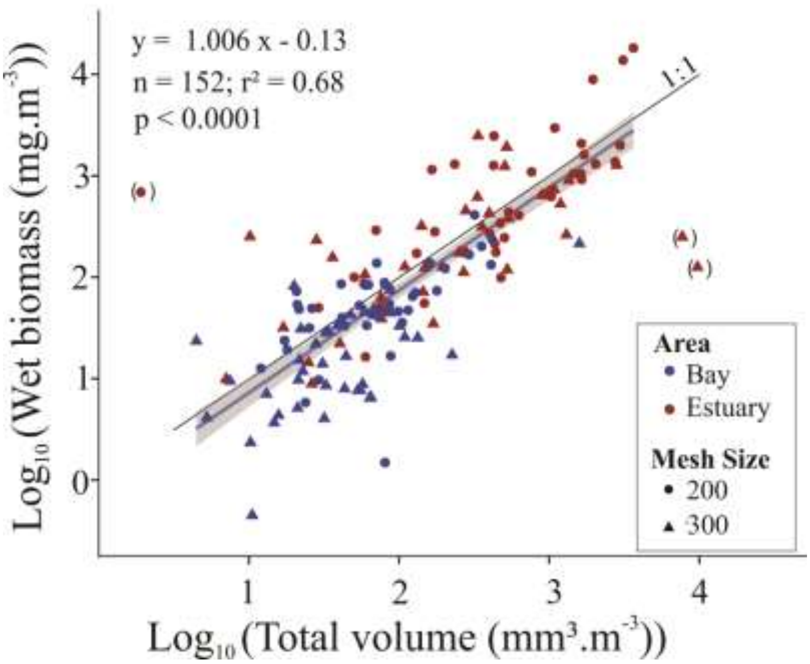

Figure 7. Relationship between $\log_{10}$ wet seston biomass (mg m^{-3}) obtained by weighing and $\log_{10}$ total volume (particles plus organisms, mm³ m^{-3}) obtained with the ZooScan. Bimonthly samples with two plankton nets (200 µm and 300 µm mesh) from 2013 to 2015 in the Rio Formoso Estuary and in the Tamandaré Bay, Brazil. *n* = 155. *Three outliers were excluded from the linear model (shown in brackets).

5.3.1 Discussion

This paper provides a novel approach to study plankton and particles in estuarine and marine ecosystems, based on image analysis. By means of separating zooplankton from suspended particles, it was possible to quantify their relative contributions as ecosystem components. This approach allows new interpretations on the composition of robust, large-sized seston in aquatic ecosystems.

5.4.1 Environmental variability

Estuaries are extremely productive habitats (FRENCH, 1997; ZARAUZ & FERNANDES, 2008) due to high concentrations of nutrients from marine, terrestrial, and anthropogenic sources. Often, these waters have a turbid appearance and a brownish color as a result of large amounts of suspended particles in the water. The low transparencies (1.2 - 3.5 m) observed in the Rio Formoso Estuary are similar to those observed in other turbid estuaries, such as the estuarine systems of Santa Cruz Channel, Brazil (FLORES-MONTES et al., 1998), San Francisco Bay, California (CLOERN, 1987), and Chesapeake Bay, Virginia (PARK & MARSHALL 2000). In contrast, seasonal nutrient-rich river plumes in the surrounding waters of the Tamandaré Bay, interchanged by oligotrophic regimes, explain the variability in its water transparency (MOURA & PASSAVANTE, 1993). The seasonal variation in temperature and salinity are within the expected range and the gradual increase in temperature during the dry season has been found by other authors for this region (REBOUÇAS 1966, NASCIMENTO-VIEIRA et al., 2010; HONORATO-SILVA, 2009).

5.4.2 Temporal variability

Higher zooplankton abundance and biomasses in the dry season in the Bay were also found by Nascimento-Vieira et al. (2010), for the same region, when the influence of rainfall, wind, and river discharge is smaller. Although reproduction peaks may occur from November to February, this is the time of the year with highest impacts from tourism activities, providing a higher influx of organic pollution. However, other studies performed in Northeastern Brazil described highest abundances during the rainy season, such as in the Taperaçu tropical estuary (Northern Brazil; Costa et al., 2008) and at the coast of Olinda (Pernambuco, Brazil; Pereira et al., 2005). Thus, total zooplankton abundance does not seem to provide a consistent response to seasonal variability in Northeastern Brazil. Further studies are necessary to investigate zooplankton variability within taxonomic groups in their abundance, biomass, and volume.

The high wet seston biomass observed in the estuary is expected, since estuaries associated with mangroves are nurseries for the marine ecosystems, with high abundances of invertebrate larvae, high primary productivity, and high input of detritus particles from the mangrove ecosystems (SCHWAMBORN & BONECKER, 1996; MELO JÚNIOR et al., 2007, SCHWAMBORN et al., 2008). Occasionally high values in the bay reflect its proximity to the reef ecosystems, which can be dominated by meroplankton larvae during reproduction events (ANGER, 2001; MACTAVISH et al., 2016), and the adjacent small estuaries (NASCIMENTO-VIEIRA et al., 2010). Similar results were also described by other authors for tropical ecosystems (MELO et al., 2002; SILVA et al., 2003; SILVA et al., 2004; MELO JÚNIOR et al., 2007). Zooplankton volume was also high during the dry season. To our knowledge, this is the first record of the zooplankton volume seasonal variability in tropical areas.

5.4.3 Sampling issues

In tropical environments, zooplankton size is relatively small when compared to temperate and boreal regions. Therefore, we expected to find higher abundances of both particles and zooplankton in the 200-µm net than in the 300-µm net (NAKAJIMA et al., 2010). Similar results were also observed by Mack et al. (2012), who compared the filtration efficiency in different sizes of the mesh nets and related that it is important to consider net-clogging effects when quantifying the zooplankton community. The zooplankton, phytoplankton, and the suspended sediments can reduce net porosity, especially those with small-sized mesh. Therefore, the Rio Formoso estuary, which is more productive in spite of its higher turbidity, appears to be more prone to net clogging than the less-productive Bay, where turbidity is lower (MACK et al., 2012). Since the reduction in net porosity could cause a significant underestimation of organisms, the smaller organisms quantified by the 200-µm net can be even higher than our estimates. These observations reinforce the ecological importance that smaller organisms play in tropical regions (PITOIS & FOX, 2006).

5.4.4 Methodological issues

This study indicates that the use of seston biomass may not be appropriate even for an approximation of zooplankton biomass, since particles make up a significant portion of the seston. This may lead to a significant overestimation of zooplankton biomass, especially in coastal and estuarine areas, which could result in misinterpretations of spatio-temporal patterns in many similar estuarine and marine areas. Similarly, in a recent study in Malaysian coastal coral reefs, Nakajima et al. (2010) found that the relative proportion between zooplankton and particles were highly variable due to the non-zooplankton contents (detritus/phytoplankton). Therefore, the estimation of zooplankton biomass from seston weight using a conversion factor does not seem feasible.

The contribution of particles to the total seston may be even higher than suggested by the present results, since many fragile particles are destroyed during net tows. It is thus not possible to estimate POM or total seston (i.e., the total mass of particulate matter in the water column) from plankton net samples, since many fragile particles are destroyed and pass through the mesh. Size range and minimum robustness of particles and zooplankton caught are mostly defined by mesh size, net diameter and towing speed. Exactly the same problem as for particles also affects the zooplankton, where soft gelatinous organisms, such as the ubiquitous ctenophores, are often destroyed and are not well resented, thus leading to an overestimation of the relative biomass of robust zooplankton (e.g., crustaceans) in zooplankton studies. Ctenophores are often destroyed by sampling, and when they are well sampled, they dissolve in formalin. Some robust organisms (e.g. copepods) may compensate this overrepresentation by their active net avoidance behavior, while others (such as brachyuran crab larvae) are very robust and cannot avoid plankton nets, being possibly overrepresented in plankton net samples. Considerable progress regarding the sampling of fragile particles has been made using devices such as the marine snow catcher (e.g. GIERING et al., 2014) or gel traps (e.g., FLINTROP et al., 2018). However, these devices are not suitable to quantify the biomass of agile living zooplankton, and do not allow for a comparison of particle and zooplankton biomass.

In spite of several methodological limitations inherent to sampling with plankton nets, image analysis allowed the discrimination between zooplankton and particles, showing the relevant contribution of particles to coastal tropical ecosystems, for both areas, especially in the estuary. These results are consistent with those found off the Californian Coast in a study based on acoustic and optical *in situ* methods (CHECKLEY et al., 2008), where particle aggregates were also more abundant than zooplankton greater than 100 µm, for nearshore areas.

5.4.5 Zooplankton and particles dynamics

A positive correlation between particles and zooplankton was also found by Ohman et al. (2012), who used a Moving Vessel Profiler (MVP) and an Underwater Vision Profiler 5 (UVP5) in association with quantitative bongo samples analyzed with a ZooScan. These authors found an increase in organic particles related to higher abundances of mesozooplankton in the California Current System. Several studies have shown that holoplankters may feed on microbes attached to particles and that predators visit aggregates because of higher prey concentrations around them (see references in Shanks and Walters, 1997).

Marcolin et al. (2013) found both higher particle and zooplankton concentrations in coastal areas when compared to oceanic stations. The particles peaks were explained by sediment resuspension and advection processes. We found more particles in the estuary in relation to the bay area, which could also be a response to resuspension processes since in the estuary the river plume should have an influence in such processes. The positive association between particles and plankton that we report in the coastal area of Pernambuco needs further studies in oceanic areas to understand plankton and particle interactions aside the coastal influence.

Mangrove sediments have the ability to retain nutrients, which become available in the nutrient cycling and thus enhances primary production, which can then be assimilated by the zooplankton, leading to high abundances (SAIFULLAH et al., 2015). Also, FLEMING et al., (1990) showed that mangroves provide a relevant source of detritus, which can be eaten by zooplankton and transferred through the food web. Previous studies showed that the zooplankton is able to ingest small particles suspended in the water column, not only from organic origin, but also plastic particles (VROOM et al., 2016). These plastic particles are also transferred via planktonic organisms to higher trophic levels through the food web and may lead to reduced growth, survival, and reproduction (SETÄLÄ et al., 2013).

Anthropogenic particles, such as microplastics, deserve special attention, due to the plethora of impacts on aquatic ecosystems (IVAR DO SUL et al., 2009; COSTA et al., 2008; IVAR DO SUL & COSTA, 2014). Indeed, microplastic are currently one of the main targets of ongoing monitoring worldwide (TEUTEN et al., 2007; COSTA et al., 2008). A study conducted by Araújo and Costa (2004) in the Tamandaré Bay, found high abundances of plastic particles in summer periods, probably related to the intense tourism activity in that region. They reported that the particles collected on the beach were from various types of plastics. In the Tamandaré Bay, Nascimento-Vieira (2000) associated the low zooplankton diversity during dry season to the impacts due to tourism, mangrove destruction, and a higher influx of organic pollution. The input of domestic sewage during the dry season is several times higher than during the rainy season, which may, in part, explain the higher abundances we found for organisms and particles. The seasonal variability of inputs of sewage and solid wastes may be a relevant factor in densely populated coastal and estuarine areas and coral reefs under anthropogenic pressure (SANTOS et al. 2015).

5.5 Conclusions

The comparison between estimates of total volume obtained through automatic measurements of images (particles+plankton) with the wet seston biomass obtained by wet weighing revealed a log-linear relationship with slope ~1 and intercept ~0. Thus, the ZooScan method provided reliable estimates of volume for both particles and plankton. This result indicates that ellipsoid volumes can be directly used to estimate the wet mass of organisms and of particles, confirming the usefulness and feasibility of the ZooScan approach and of the use of ellipsoid volumes based on scanned images.

An overestimation of *in situ* living biomass by simply weighing plankton samples has often been suggested. This would be caused by the residual mass of interstitial water that remains between organisms when using this method. No such effect of interstitial water was detected here, even though no pumps or other destructive devices were used to remove interstitial water, as has been speculated and recommended by several authors (*e.g.,* JACOBS & GRANT 1978; HARRIS et al., 2000). Yet, an overestimation of wet biomass does occur, but it is due to the presence of non-living particles, that are generally neglected in standard analyses of plankton net samples. Particles always composed a huge part of the samples volume (*i.e.,* wet mass), and it was often the dominant component.

Wet weight is clearly not an appropriate method for the estimation of zooplankton biomass, at least not in tropical coastal and estuarine areas. Wet weight quantifies the mass of the total non-fragile, large-sized seston, *i.e.*, robust mesozooplankton and robust mesoparticles. However, it is unclear how to use and interpret such a combined and mixed parameter in the study of plankton. In conclusion, this study shows the extent of the importance of particles for wet weight data obtained with regular plankton nets, and the usefulness of estimating biomass through simple, quick, and precise image analysis.

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6 ARTIGO 2: A FRESH LOOK AT MICROPLASTICS AND OTHER PARTICLES IN THE TROPICAL COASTAL ECOSYSTEMS OF TAMANDARÉ, BRAZIL

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Abstract

Plankton organisms, biogenic particles, inorganic mineral particles, and microplastics are the four main components of particulate organic matter in aquatic ecosystems. We propose a new index, the Relative Microplastics Concentration (RMC, in %), considering that microplastics are more deleterious when food is scarce. A total of 112 plankton net samples were collected in estuarine, coastal and shelf environments of Tamandaré, Brazil. Particles were identified by image analysis (ZooScan) and FTIR. Higher concentrations of total microplastics, PP (Polypropylene) and PE (Polyethylene) in the estuary indicate an oceanward decreasing gradient from terrestrial sources. Higher concentrations of nylon fibres were found offshore. Yet, RMC indicated that the Bay had the most severely impacted ecosystems (RMC: 2.4% in the estuary, 5.1% in the Bay, and 2.0% on the shelf), for total microplastics and PP & PE. Shelf ecosystems were most severely impacted with nylon fibres. RMC analysis provided a new perspective into the impact of microplastics on tropical coastal food webs.

Keywords: Polypropylene; Polyethylene; Nylon; Relative Microplastics Concentration; ZooScan; FTIR.

6.1.1 Introduction

Plankton organisms, biogenic particles, inorganic mineral particles, and microplastics are the four main components of particulate organic matter in aquatic ecosystems (Fleming et al., 1990; Bowers and Binding, 2006; Nakajima et al., 2010; Vroom et al., 2017; Lins Siva et al., 2019). However, there are no published studies available yet, which consider these four components in a synoptic way and compare their distribution patterns.

Suspended particles are key elements in marine ecosystems, mainly because of their role in fueling food webs and biogeochemical cycles, such as the biological “carbon pump”

(Schumann and Rentsch, 1998; Schwamborn et al., 2002; Schwamborn et al., 2006; Checkley et al., 2008). Recent studies showed that non-organismic particles (particles that do not constitute a single living organism, e.g., detritus, aggregates, sand grains and microplastics) constitute a significant portion of common plankton samples, leading to an overestimation of plankton biomass in the oceans (Nakajima et al. al., 2010; Ohman et al., 2012; Lins Silva et al., 2019). In estuarine and coastal waters, ignoring the huge contribution of particles may lead to a severe underestimation of seston biomass by traditional wet weight-based methods (Lins Silva, et al., 2019). Coastal tropical environments, such as mangroves and coral reefs, receive many terrigenous materials. Also, nearshore coastal ecosystems may show high concentrations of anthropogenic particles, such as microplastics (Chong et al., 2001; Barnes et al., 2009).

Zooplankton, biogenic particles and microplastics are traditionally studied by means of sampling with fine-meshed nets and manual counts under a microscope (Blanchot et al., 1989; Neumann-Leitão et al., 1998). Since manual sorting, identification, and quantification of particles and plankton are a time-consuming task, image analysis has become a popular tool in the last ~30 years. Benchtop imaging devices, such as the ZooScan (Grosjean et al., 2004), provide good quality images, with almost perfect focus. Fourier-Transformed Infrared Spectroscopy (FTIR) can be used to identify polymers and to investigate the chemical composition and weathering of microplastics (Dutra et al., 1995, Munajad et al., 2018). Extensive protocols for the preparation of microplastic samples have been developed, including slow and complex dehydration procedures, to allow a reliable interpretation of FTIR spectra (Dutra et al., 1995, Pinho and Macedo 2005). Different sample preparation techniques have been applied, since the methods to separate polymers from other components depend on the diversity of particle types (Dutra et al., 1995, Pinho and Macedo 2005, Munajad et al., 2018). Few studies have used FTIR to analyse microplastics taken from plankton net samples (Di Mauro et al., 2017; Cincinelli et al., 2017), and none have yet combined ZooScan and FTIR to distinguish plankton organisms, biogenic particles and microplastics.

Although there are few studies that estimated the chemical composition and characterization of these particles (McCave et al., 2001; Cincinelli et al., 2017), there are no studies about the origin (anthropogenic or natural) and type (chemical markers) of suspended particles in tropical marine environments.

In spite of the vast recent literature on this subject, there is no practical approach available for the assessment of contamination with microplastics, that explicitly considers the relative contribution of these pollutants, with regard to the available food (suspended particles and plankton) in the water column. This is probably due to the fact that most studies on microplastics destroy and digest biogenic particles and plankton in the samples with acids or enzymes, previous to counting microplastics. The few studies that actually consider microplastics / zooplankton ratios (Cole et al., 2013; Botterell et al., 2019), but do not consider non-organismic biogenic particles, such as plant detritus (Schwamborn et al, 2006), carcases (Jonathan da Silva et al., 2020), and marine aggregates (Kvale et al., 2020).

In the present study, we suggest a new approach and a new index (RMC) to analyze contamination with microplastics. Furthermore, we used this new approach to test the hypothesis that particle type, concentration and volume (in absolute and relative units), differ between ecosystems (mangrove estuary, coral reef-lined bay and continental shelf), thus helping to reveal sources and sinks of biogenic and anthropogenic particles. Also, we aim at discerning the ecosystems that are most impacted by different types of microplastics.

6.2 Methods

6.2.1 Study area

The sampled areas range from highly turbid, “brown” estuarine waters, lined by mangroves, to clear “green“ water nearby coral reefs, and oligotrophic “blue“ waters at mid-shelf. The Rio Formoso Estuary (8° 39' - 8° 42′S and 35°10' - 35° 05′W) extends over 12 km and along its route, it receives wastes from domestic sewage and sugar cane industry (Fidem, 1987; CPRH, 1998). The Rio Formoso Estuary is located ~4 Km North of the Tamandaré Bay. The estuarine channels are entirely bordered by mangroves with muddy sediments that are rich in organic matter, which appear to be the most important source of suspended matter in the estuarine area (Silva, et al., 2003; Vasconcelos et al., 2004).

Tamandaré Bay (8° 44' - 8° 47′ S and 30° 0.5' - 35.07′ W) is a coastal embayment lined by several parallel sandstone reefs with high sedimentation rates and low hydrodynamics, which promote water imprisonment, functioning as an open coastal lagoon (Rebouças, 1966; Camargo et al., 2007). The coastal dynamic is influenced by three large rivers: Rio Formoso, which drains from the north, and Mamucaba and Una rivers, draining into the Bay from the south. Intertidal reef tops are predominantly covered by zoanthids, calcareous algae, and hydrocorals, with several endemic coral species (Amaral and Ramos, 2007; Santos et al., 2015). The main economic activities in this region are agriculture (mostly sugar cane plantations), tourism and fisheries (Moura and Passavante, 1993; Araújo and Costa, 2004). Marine erosive processes observed in this area are related to anthropogenic interventions, such as urban expansion (CPRH, 1998).

The continental shelf off Pernambuco is relatively narrow (~35 km wide), with shallow depths. The shelf break is at ~50 meters depth, with warm waters, high salinity and a sedimentary base composed of biogenic, terrestrial and carbonate sediments (Manso et al., 2003). The adjacent inner shelf off Tamandaré has a sandstone linear beach rock parallel to the coast, which is a substrate for the development of algae and corals, and also an effective protection against wave energy (Manso et al., 2003; Camargo et al., 2007).

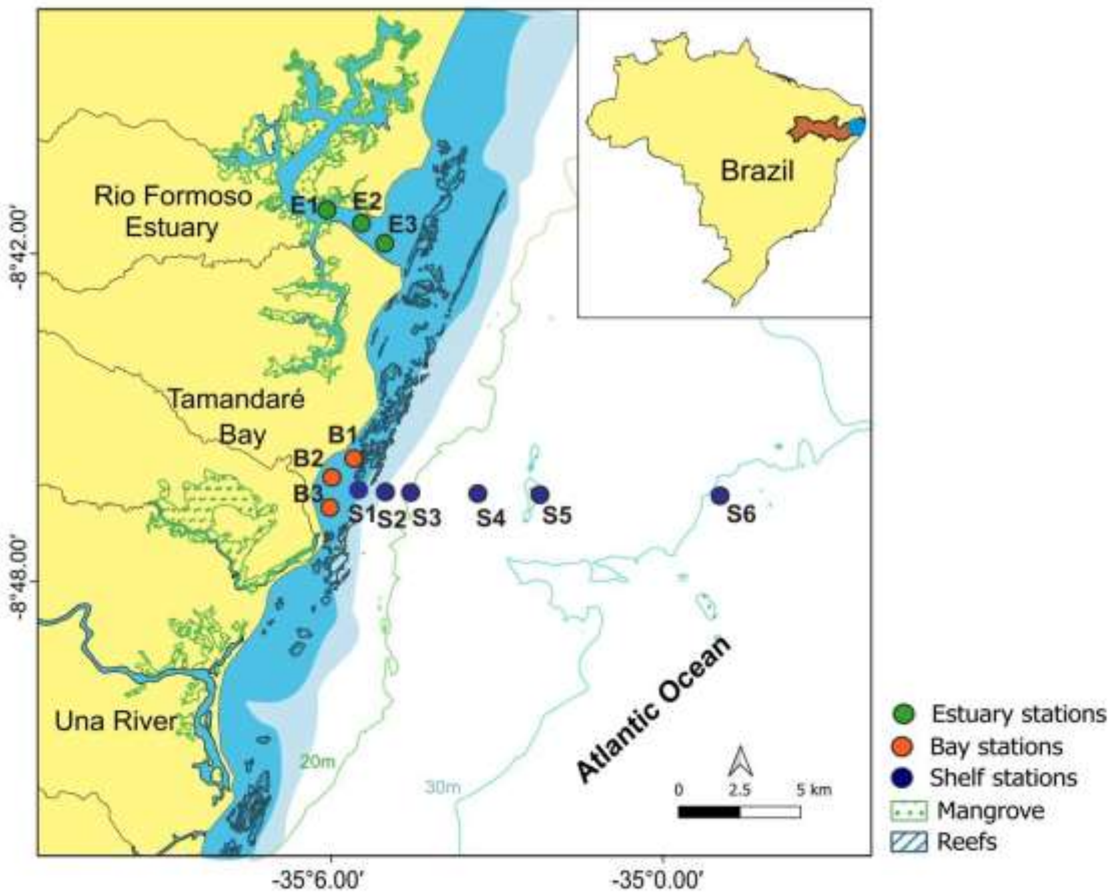

Fig. 1. Map showing the study area and stations where sampling was conducted in three environments (Rio Formoso Estuary, Tamandaré Bay, and on the adjacent continental shelf),

from April 2013 to May 2015. Map inlet (above): map of Brazil showing the State of Pernambuco (dark brown) and the location of the Tamandaré coastal region (blue circle). Plankton samples and abiotic parameters were obtained bimonthly between April 2013 and May 2015. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

The rationale for the choice of the study areas was to investigate three tropical coastal ecosystems (a highly turbid mangrove estuary, a coral reef-lined bay, and a nearshore shelf area) that may receive considerable amounts of anthropogenic particles and other pollutants, but are not located within any atypically polluted geographical setting. These three areas were chosen since they are relatively pristine (i.e., compared to other coastal areas, such as coastal megacities), without any large cities or large industrial complexes nearby. They are included within two large Marine Protected Areas (State Decree, nº 19.635, March 13, 1997 and Federal Decree, s/n, October 23, 1997).

The climate is hot and humid, with distinct rainfall seasons and wind energy patterns. Wind speed at sea level exhibits a characteristic seasonality (Silva et al., 2011), with a distinct windy season from July to October (strong SE to E winds) and a calm season from January to May (weak north-easterlies). Rainfall is more intense between March and August, with peaks in July, i.e., the rainy season, while the dry season is generally from September to February (Ferreira et al., 2003; Grego et al., 2009; Venekey et al., 2011).

6.2.2 Plankton sampling and hydrological parameters

A total of 112 plankton samples were analyzed. Sampling occurred in regular bimonthly intervals, during two years, between April 2013 and May 2015 during ESPLAN and INCT AmbTropic campaigns. During each field campaign, twelve fixed stations were sampled, three in the Rio Formoso Estuary, three in Tamandaré Bay, and six on the continental shelf, distributed along a straight transect (three stations at the nearshore shelf and three at mid-shelf), from the reef line to the mid-shelf (Fig. 8). Tows were performed using a conical-cylindrical plankton net with a 300-μm mesh (diameter: 60 cm), by means of subsurface horizontal tows during 5 min at a speed of 2–3 knots, during ebb (Rio Formoso Estuary) and flood tides (Tamandaré Bay and Shelf). The upper rim of the net opening was maintained at the surface with a float (sampling depth: 0 to 0.6 m). Sampling was conducted during daytime, at the same tidal phase, during new moon spring tides. A calibrated flow meter (Hydro-Bios, Kiel) was coupled to the mouth of the net to estimate the filtered volume. Samples were immediately preserved in formaldehyde (4% final concentration), buffered with sodium tetraborate (5 g L^{-1}), as described by Omori and Ikeda (1984). A CTD probe (CastAway, YSI) was used to obtain vertical profiles of salinity and temperature. Water transparency was estimated from the Secchi-disk depth (Preisendorfer, 1986).

6.2.3 Image acquisition and analysis

A ZooScan device (Hydroptic model ZSCAN03) was used to digitise the plankton samples with 2400 dpi resolution, following the protocol established by Grosjean et al. (2004; http://www.zooscan.obs-vlfr.fr/). Each sample was separated into two size fractions (> 1000µm and < 1000µm) to avoid underestimating large organisms and particles, considering that large objects are less abundant (Gorsky et al., 2010). Samples obtained in the Rio Formoso Estuary and in Tamandaré Bay were diluted in filtered water in a beaker, according to the total number of particles in the sample, and then carefully mixed before the extraction of a 10 ml fraction, to obtain a sub-sample of approximately 2,000 particles for subsequent scanning (Grosjean et al., 2004). For samples obtained on the continental shelf, a Motoda box splitter was used (Motoda, 1959). The number of objects contained in each scanned fraction ranged from 40, for the size fraction > 1000µm, up to 8,000 objects, for the size fraction < 1000µm.

Images were processed using the ZooProcess software (Version 7.25), written in the ImageJ macro language (https://imagej.nih.gov/ij/), developed for plankton image analysis. The Plankton Identifier (PkID) software (version 1.3.4), was used for semi-automatic classification. First, we built a training set, wich was then used for Random Forest, an algorithm for the automatic classification of vignettes into predefined categories. Finally, all vignettes were manually validated to correct for misclassification. Size parameters were converted from pixels to micrometers, according to the scanner resolution (size of 1 pixel at 2400 dpi: 10.58 µm). To minimize possible contamination of the samples on board and in the laboratory, we applied a series of protocols and blanks, e.g., we used filtered water (20 micrometer mesh filters), and all sample flasks and materials were thoroughly washed before placing the sample. All particles were classified according to their shape and gray levels, based on the ZooScan images (vignettes). For the classification according to gray level, three different categories were created: dark, opaque and transparent particles, whose shape could be globulose or flat.

Particle volume (mm^3) was usually estimated as the ellipsoid volume, based on the lengths of major and minor axes of the equivalent ellipse (i.e., an ellipse with the same area and similar height/width ratio as the original vignette, Vandromme et al., 2012 and Stemmann and Boss, 2012), except for flat particles. Flat particles were considered as having a flat shape and their volume (mm^3) was calculated based on the surface area (the "area_exc" parameter, in mm^2) multiplied by the thickness (mm) of each particle type (Grosjean et al., 2004). The thickness was measured under a stereomicroscope (Zeiss, Stemi SV6 model) in 30 randomly chosen plankton samples. For each sample, 50 particles were taken from three different categories (opaque, dark, and transparent flat particles), and classified according to their gray level. For opaque and dark flat particles, mean thickness was 781 µm, whereas for transparent flat particles, mean thickness was 319 µm (Lins Silva et al., 2019).

6.2.4 Fourier-Transformed Infrared Spectroscopy analysis (FTIR)

The specific density of plankton and biogenic particles has a broad range, but usually lies between 1.03 and 1.10 $g.L^{-1}$ (Goldemberg et al., 2010). On the other hand, man-made polymer particles have a much narrower range of specific density values (Mark, 1999). The density of these polymers varies only little over time, e.g., their buoyancy may decrease due to rapid colonization with microorganisms (Cole et al., 2011). The most common polymers, which are are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyamide (PA), polyethylene terephthalate (PET), polyvinyl alcohol (PVA). They present specific ranges of density values: PE: 0.93 to 0.98 $g.L^{-1}$, PP: 0.89-0.91, PS: 1.04-1.11, PVC: 1.2-1.45, PA: 1.13 to 1.5, PET: 1.38-1.39, PVA: 1.19-1.35. (Avio et al., 2016). Thus, their density can be useful to separate biogenic particles and microplastics.

The particles preparation had two objectives: to separate biogenic particles from microplastics according to their density, and to remove water from microplastics. Vacuum filtration (Whatman cellulose acetate filters 1.2 µm pore size) was used to remove the particles from the original sample. Samples were put in a separatory funnel (100 ml of solution + particles), in contact with controlled density liquids, mixed and let to rest for 30 min. The liquids were: ethanol (C_2H_5OH) + methanol (CH_3OH) + sodium bromide solution (NaBr) solution (0.88 g/cm^3), ethanol + sodium chloride (NaCl) solution (P.A – A.C.S; 0.81 g/cm^3), and absolute ethyl alcohol (P.A – A.C.S; CH_3CH_2OH.; 0.79 g/cm^3). The decanted fraction passed to the next step. The supernatant fraction was dried in a dry oven at 50°C for twenty-four hours to six months, depending on the water contents in the sample. After that, additional two steps were realized intending to dissolve any polymer particle which was not removed from biological particles in the previous three steps. Particles went into two solvents: acetone (P.A – A.C.S; $CH_3(CO)CH_3$ 0.78 g/cm^3), and chloroform (P.A – A.C.S; $CHCl_3$,1.48 g/cm^3). Again the decanted fraction and supernatant fraction were dried in a dry oven at 50 °C for periods that varied from twenty-four hours to six months, depending on the water content of the sample.

FTIR was used to analyze the typology of all biogenic and abiogenic (*i.e.*, microplastics) particles that were previously classified with the ZooScan. For this purpose, a subset of 30 plankton samples (10 samples randomly chosen from each study area), was selected to allow a detailed verification of the typology of all particles (biogenic particles and microplastics). A Shimadzu Fourier Transform Infrared spectrophotometer, model Prestige 21, with a diffuse reflectance module, was used to acquire the infrared spectra for all samples. We performed 24 scans per sample, with 4 cm^{-1} resolution, according to the ATSM SP E1252 – 98 normative. All spectra were treated using the OriginPro 8 software. The identification of the polymers was made by comparing the sample spectra with standard spectra contained in the Hummel Polymer and Additives library (Silverstein, et al., 2007). FTIR was also used to evaluate the weathering status of particles (fresh *vs* weathered polymers).

6.2.5 Statistical analyses

ZooScan data and images were used for quantifying concentrations (counts per cubic meter) and volumes (mm^3 per cubic meter) of zooplankton, microplastics and other particles. Additionally to quantifying absolute concentrations, we propose a new index of microplastics contamination, the Relative Microplastics Concentration (RMC, in %). RMC is calculated as

$$\mathbf{RMC\ (\%) = 100 * (\ m / (z+p+m)\ )},$$

where m = microplastics concentration, z = zooplankton, and p = “other particles” (e.g. biogenic particles detritus, sand grains, etc.). The rationale behind the proposed RMC is that the relative concentration is an index for the probability of encountering microplastics by any organism feeding in the water column at a given location. Also, it provides a straightforwardly intelligible percentage of microplastics among all food particles (living and nonliving) available for planktivores.

All data sets were tested for normality and homoscedasticity using Kolmogorov-Smirnov and Levene tests, respectively, prior to analysis. Since the data sets were not normally distributed, even after log10(x+1) transformation, non-parametric methods were applied to the 11 particle types tested and key parameter ratios, such as the ratio "total microplastics" / "total zooplankton" and RMC. A multivariate two-way PERMANOVA (Anderson, 2001) was used to test for significant effects of three factors: i) seasons (dry *vs* rainy), ii) areas (Estuary, Bay and Shelf) and iii) their interaction (seasons *vs* areas). For each particle type, a separate PERMANOVA was conducted (10,000 permutations), based on an euclidean distance matrix, (Anderson, 2017), applying the function "adonis" within the "vegan" R package (Oksanen et al., 2017). For variables that displayed significant PERMANOVA results for the factor “study areas” ($p < 0.05$), *post-hoc* tests for pairwise multiple comparisons of mean rank sums (i.e., non-parametric Kruskal-Nemenyi tests, Nemenyi, 1963, Hollander and Wolfe, 1999, Demsar, 2006), were conducted. These pairwise K-N tests (e.g., “Bay *vs* Estuary”), were conducted using the function “posthoc.kruskal.nemenyi.test” in the R package “PMCMR” (Pohlert, 2020). All analyses were conducted within the R environment for statistical computing and graphics, version 3.6.3 (R Core Team, 2020).

6.3 Results

6.3.1 Hydrographic features

The three study areas (Rio Formoso Estuary, Tamandaré Bay and Continental Shelf) showed characteristic hydrographic features (Fig. 9). Significantly ($p < 0.0001$) lower transparency was found in the Estuary (Secchi depth range: 1.2 – 3.5 m; mean: 1.8 m) than in the Bay (1.0 - 5.5 m; mean: 3.1 m) and on the Shelf (1.0 - 19.0 m; mean: 5.3 m). Euhaline conditions were found in the Bay (salinity range: 35.0 - 36.5; mean: 35.9) and on the Shelf (33.8 - 37.4; mean: 36.3). Conversely, mesohaline to euhaline conditions were observed in the Estuary (27.0 - 36.4; mean: 32.1). Temperature and salinity varied seasonally, with a gradual increase during the dry season, from September through February (Suppl. Table 1).

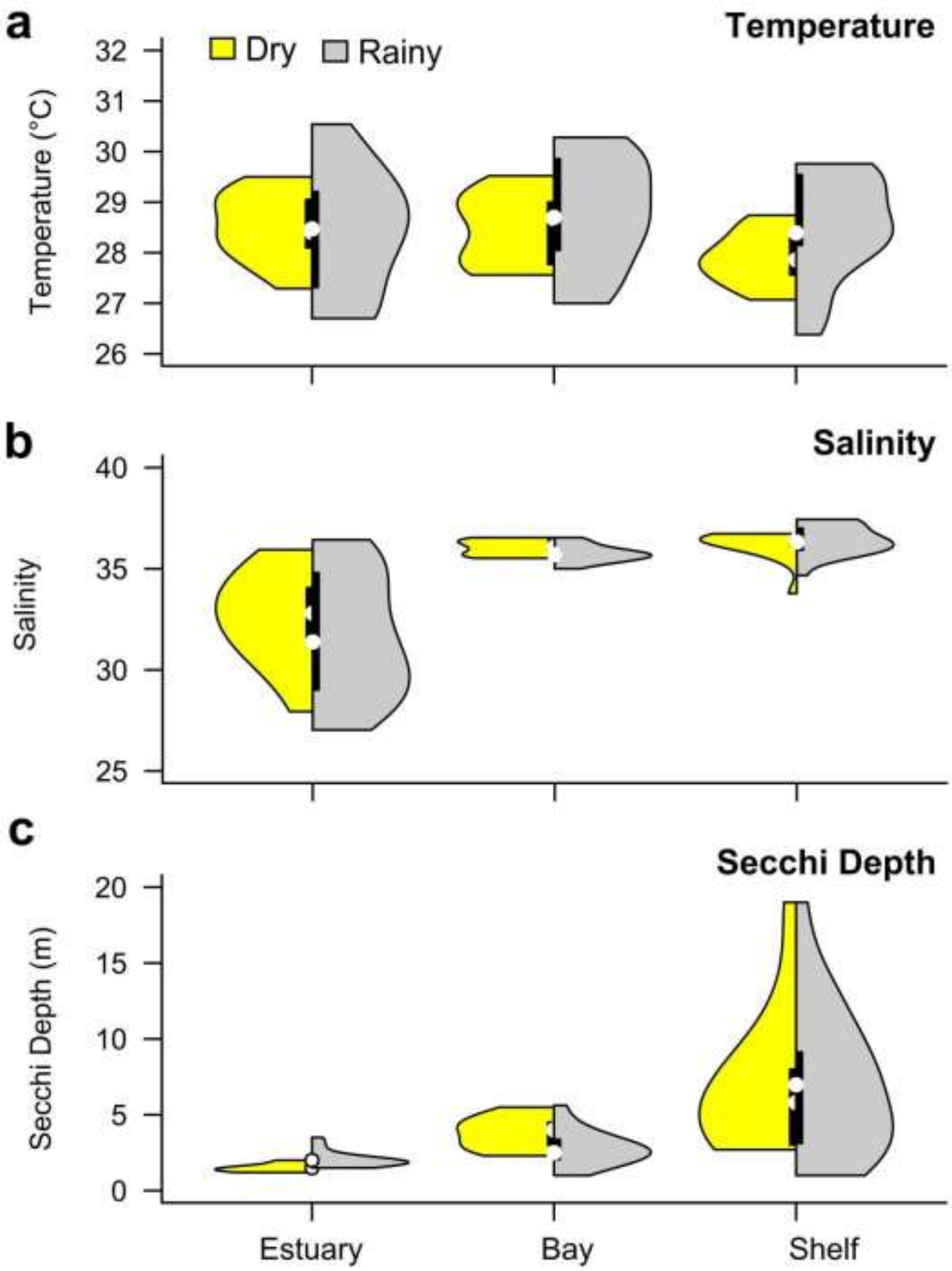

Fig. 2. Vioplots (Kernel density distributions with boxplots) of environmental variables (salinity, temperature, and Secchi depth) in estuarine, bay and shelf waters. Yellow color represents the dry season and gray color represents the rainy season. Bimonthly sampling was conducted from 2013 to 2015 in the Rio Formoso Estuary, Tamandaré Bay and on the adjacent Continental Shelf. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) Estuarine waters were often brown and turbid, and transparency was significantly lower ($p = 0.0006$) in the rainy season than in the dry season, when particles, macroalgae and DOM (Dissolved Organic Matter) were more abundant. In the dry season, there was an intrusion of offshore transparent waters into the estuary. In the Estuary, temperature and salinity did not differ significantly between seasons (dry *vs* rainy).

In the Bay, water transparency was significantly higher in the dry season ($p = 0.02$), when low wind intensity and low rainfall led to calm, transparent "blue" waters. Conversely, "green" waters (Secchi depth range: 1.5 - 4.5 m, mean: 3.0 m) were dominant during the rainy season, due to nutrient inputs from stronger wind turbulence and increased runoff from adjacent rivers. On the Shelf, water transparency was highly variable, but often showed transparent, "blue" waters, with > 5 m Secchi depth.

6.3.2 Particle classification

The 112 samples analyzed contained plankton and twelve types of non-organismic particles (biogenic particles and microplastics (MP), see Suppl. Table 2). Particle size varied from 299 μm to 57 mm equivalent spherical diameter (ESD). FTIR spectroscopy allowed us to distinguish and typify four types of particles that were common in all sampling areas: i.) biogenic particles (vascular plant detritus, fragments of macroalgae, marine aggregates and exuviae), ii.) polypropylene (PP), iii.) polyethylene (PE), and iv.) polyamide (i.e., nylon fibers). Even though we detected and distinguished polypropylene and polyethylene particles with FTIR, we pooled these two categories (PP & PE) for our quantitative analyses, because they were not distinguishable in the ZooScan images. Also, when it was not possible to relate the ZooScan images with the infrared absorption spectrum with FTIR the classification of some types of particles was kept in the gray levels (see Suppl. Table 2).

Polymeric particles displayed well defined FTIR spectral peaks and bands, which allowed their identification, even when the particle showed extra peaks due the ageing process. Weathering bands were present to some extent in all polymer samples. There was no detectable contamination with fresh nylon (e.g., from the plankton nets). Conversely, the FTIR spectrum for biogenic particles showed persistent water bands and small peaks which can be considered as the resultant spectra from the sum of several compounds with complex chemical structure, consistent with biological samples (Suppl. Fig. 1).

Nine categories of non-zooplankton particles (Suppl. Table 2) could be classified and described according to shape and grey level descriptors, using digital images obtained with a ZooScan equipment: i.) flat dark particles, ii.) flat opaque particles, iii.) transparent particles, iv.) opaque particles, v.) marine aggregates, vi.) fragments of macroalgae, vii.) exuviae/carcasses, viii.) sand grains, and ix.) fibers. Multiples (touching objects) were not included in this list, since they are often composed of zooplankton organisms and aggregates. These categories of particles were based on grey level distributions and a matrix of shape descriptors. The combination of FTIR and Zooscan analyses allowed us to refine the identification process (PP & PE, nylon fibers, vascular plants), increasing the number of categories from nine to twelve (see Suppl. Table 2).

6.3.3 Distribution of microplastics and other particles

Three main groups of particles were found. The most abundant group was composed of biogenic particles (i.e., derived from vascular plant detritus, exuviae, carcasses, and marine aggregates), followed by opaque particles (origin unknown), and microplastics (e.g., nylon fibers, polyethylene and polypropylene - PP & PE) (Fig. 10). Non-zooplankton particles were abundant in all environments (Estuary, Bay and Shelf), usually comprising more than 50% of the plankton samples.

The concentration of particles generally decreased towards offshore, with high variability within areas. Total particle concentration ranged from 13.4 to 4903.7 particles m^{-3} in the Estuary, from 6.9 to 525.5 particles m^{-3} in the Bay and from 0.95 to 206.3 particles m^{-3} on the Shelf (Suppl. Table 3). While there was a striking and consistent effect of the spatial gradient, no consistent seasonal effects were detected for the total concentration of particles.

In the Estuary, the most abundant particles were vascular plant detritus (47% of all non-organismic particles, mean percentage, in units of concentration), most likely originating from the mangroves, followed by opaque (21%) and transparent particles (14%). In the Bay, the most abundant particles were opaque (25%), transparent (20%) and PP & PE (14%).

On the Shelf, marine aggregates (i.e., complex particles formed though coagulation of gelatinous detritus and plankton) were the dominant type of particles (35% of total particle concentration, on average), followed by vascular plant detritus (21%) and opaque particles (18%). Microplastics were also very abundant (14%) in Shelf waters (Fig. 10).

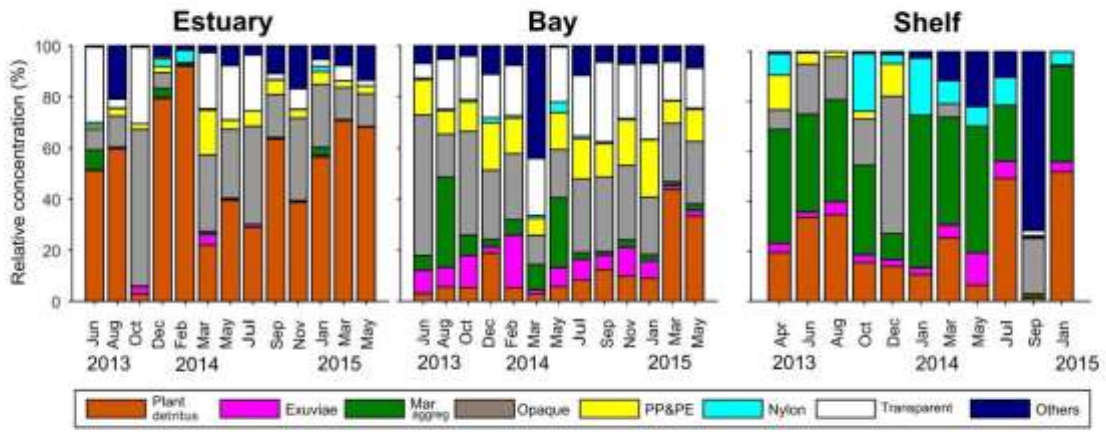

Fig. 3. Relative concentration of suspended particles in three areas (Rio Formoso Estuary, Tamandaré Bay and Continental Shelf) off Tamandaré,Brazil, n = 112.

Significant effects of the factor "area" (Estuary vs Bay vs Shelf) were detected for the relative concentration of all particle types. Highest median concentration values were found in the Estuary, followed by Bay and Shelf waters ($p < 0.001$, PERMANOVA, Suppl. Table 4). The *post-hoc* pairwise tests revealed significant differences between areas in absolute concentration of most particle types (Suppl. Table 4).

6.3.4 Contamination with microplastics

Total concentrations of microplastics (PP & PE and Nylon, together) were highest in the Estuary, and decreased oceanward (Fig. 11a), similarly to other particles (Fig. 11b), such as biogenic particles (plant detritus, macroalgae, marine aggregates and exuviae), sand, opaque, opaque flat, transparent and transparent flat particles. However, PP & PE and nylon displayed marked differences in their spatial variability, reflecting their different origins and sinks. The concentration of PP & PE, which were the most abundant microplastics, had a maximum in the Estuary and decreased continuously towards the open ocean (Fig. 11c). Conversely, nylon fibers also showed maximum concentrations in the estuarine area, but higher concentrations at mid-shelf than in the Bay area (Fig. 11d). The concentration of zooplankton, similarly to the concentration of microplastics and the other particles, was highest in estuarine waters and decreased towards the Shelf (Fig. 11e). The Relative Microplastics Concentration (RMC, in %) had maximum values in the Bay and nearshore Shelf areas, and lower RMCs in the Estuary. Minimum RMC values were found at the offshore shelf (Fig. 11f).

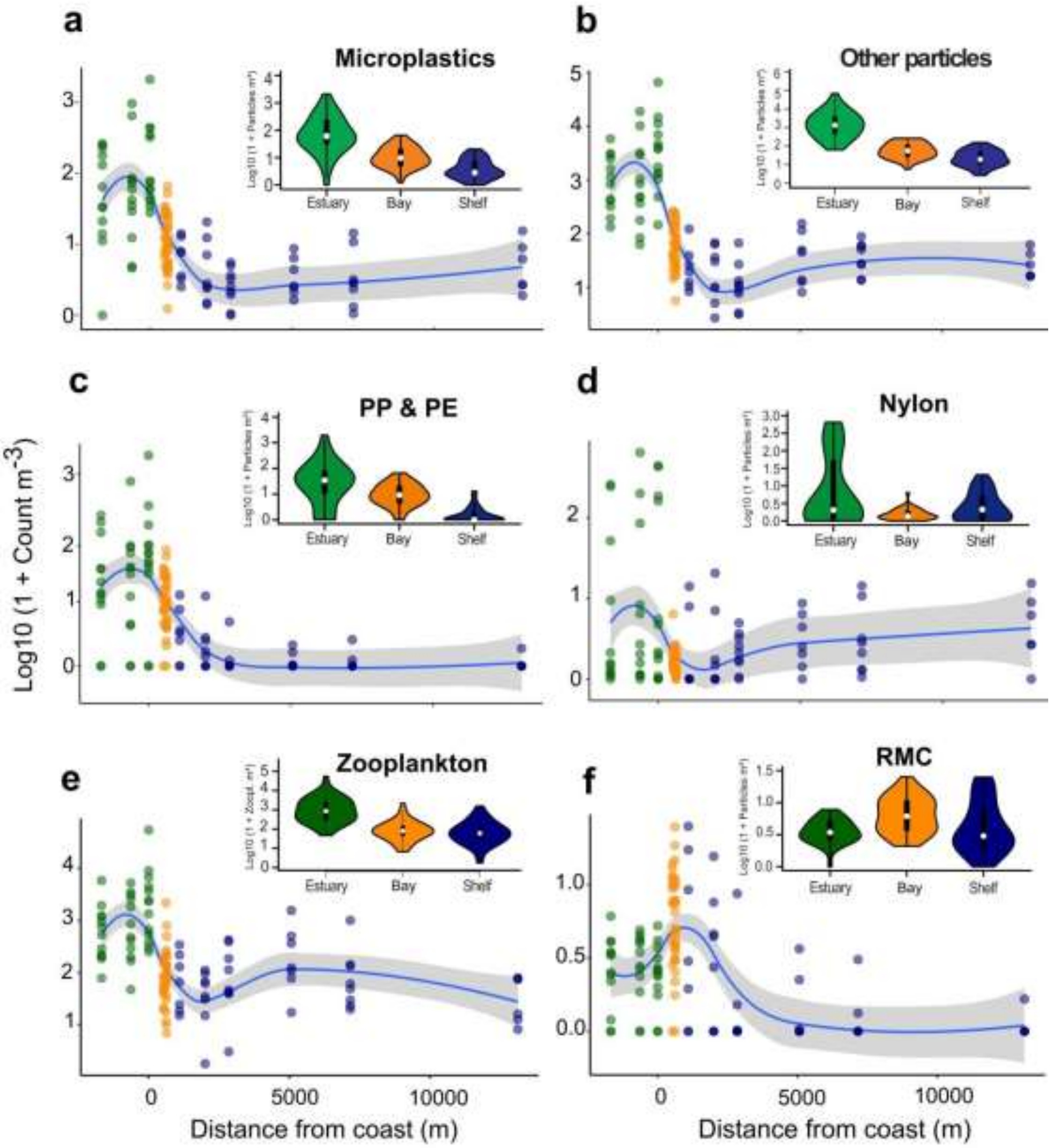

Fig. 4. Gradient of spatial distribution of the concentrations (counts m- ³) of total microplastics (a), other particles (plant derived + marine aggregates + macroalgae + exuviae + sand + opaque + opaque flat + dark flat + transparent + transparent flat) (b), PP & PE (polyethylene + polypropylene) (c), and Nylon (d), total zooplankton (e) and Relative Microplastics Concentration (RMC, %) = 100 * total microplastics/other particles + zooplankton + microplastics (f). All values have been log10(x+1) transformed prior to plotting.

The study areas differed with regard to RMC (PERMANOVA, $p = 0.004$). Highest RMCs were recorded in the Bay (Fig. 12a), indicating a higher impact with microplastics in relation to the Estuary and Shelf. Significant differences were not observed for the relative volume of microplastics (RMV) between the areas (Fig. 12b), probably by volume calculations. For the microplastics ratio (PP & PE and Nylon)/other particles (in concentration units), there was a highly significant effect of the factor "area" (PERMANOVA, $p = 0.001$), with significantly lower proportions ($p < 0,001$) in the estuary and similar rates in the bay and shelf areas (Fig. 12c).

When testing the microplastics / zooplankton ratio (in concentration units), a significant effect of the factor "area" (PERMANOVA, $p = 0.013$) was observed, with highest values also being recorded in the Bay (Fig. 12d), similarly to RMC.

For the PP & PE / other particles + zooplankton + microplastics ratio (in concentration units), there was a highly significant effect of the factor “area” (PERMANOVA, $p = 0.001$) with highest values in the Bay, followed by Shelf and estuarine stations (Fig. 12e). Nylon fibers had a different spatial pattern. When testing the nylon / other particles + zooplankton + microplastics ratio (in concentration units), the highest relative concentration of nylon fibres was found in the Shelf area (PERMANOVA, $p = 0.002$; Fig. 12f).

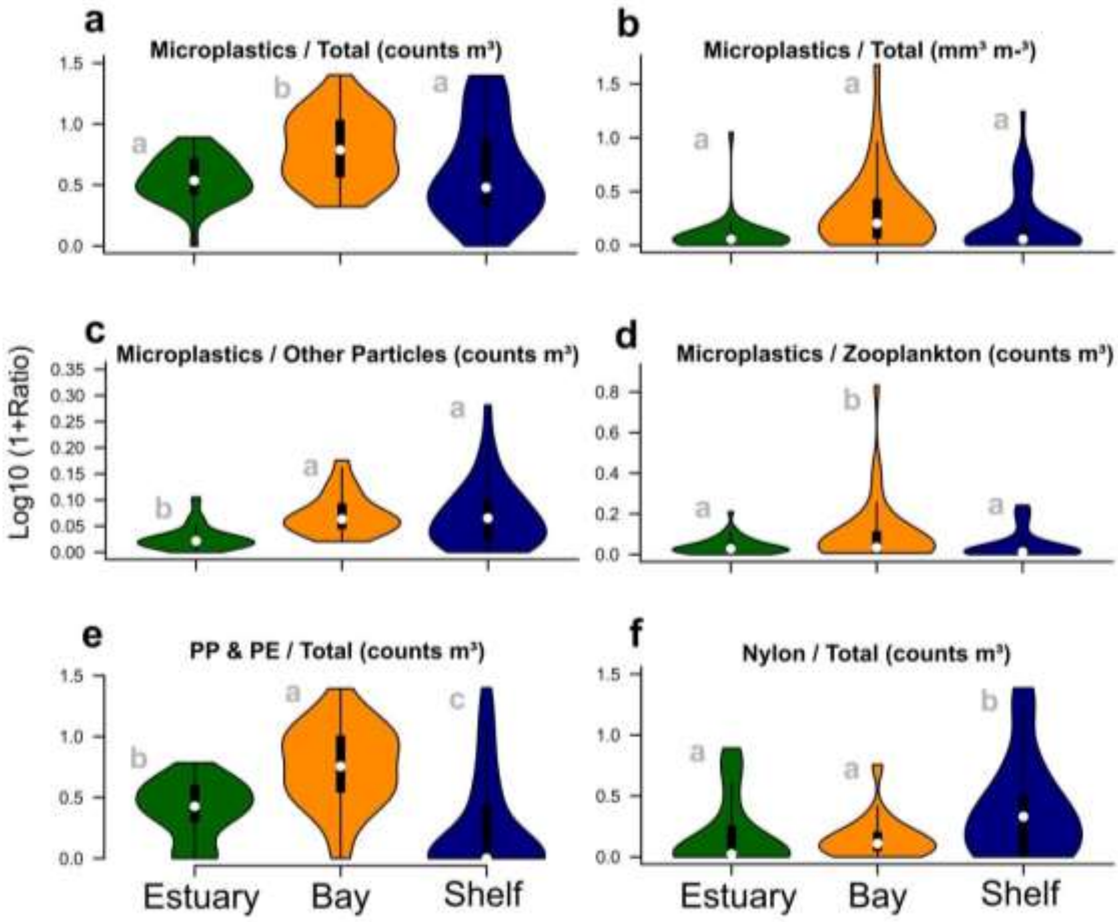

Fig. 5. Indices of microplastics contamination in units of concentration (counts m- ³) and volume (mm³ m- ³). Microplastics: Nylon + PP & PE; Total: other particles + zooplankton + microplastics; Other particles: non-plastic and non-zooplankton particles. Other particles are represented by plant derived detritus, marine aggregates, macroalgae, exuviae, sand, opaque, transparent, dark flat, opaque flat and transparent flat particles.

6.4 Discussion

This study provides new insights into the distribution of microplastics and other suspended particles in tropical estuarine and marine environments, combining two different optical methods (ZooScan and FTIR). Through the use of a new approach and new indices, we quantified the contributions of different types of particles in the meso (>299 µm) size range, natural or not, as ecosystem components. This approach allows novel interpretations of the composition of robust, large-sized seston in the aquatic ecosystems. Also, the use of a new index (RMC) provided a new perspective into the impact of microplastics on the food particle spectrum available for planktivorous organisms, and consequently, on estuarine and marine ecosystems.

6.4.1. Hydrography

Estuarine areas are extremely productive habitats (French, 1997; Zarauz and Fernandes, 2008) and estuarine waters often have a turbid appearance and a brownish color as a result of large amounts of suspended particles, such as mangrove leaf detritus (Schwamborn et al., 2002, Schwamborn et al., 2006, Lins Silva et al., 2019). The low transparencies observed in the Rio Formoso Estuary are similar to those observed in other turbid estuaries, such as the estuarine system of Santa Cruz Channel, Brazil (Flores-Montes et al., 1998; Schettini et al., 2016). The seasonally varying mixture of nutrient-rich river plumes and oligotrophic shelf waters explain the variability in water transparency regimes of Tamandaré Bay (Maida and Ferreira, 1995; Santos et al., 2010). On the adjacent continental shelf, water transparency was generally much higher and extremely variable, with visibilities ranging from 1 to 19 meters, depending on seasonal variations in rainfall and sediment resuspension driven by wind-induced turbulence (Rebouças, 1966; Moura and Passavante, 1993).

6.4.2 Spatial heterogeneity of microplastics and other particles

In this study, maximum values of total particle abundance and volume in the Estuary showed the importance of substantial inputs of detritus of riverine origin (Williams and Simmons 1997). Continental shelf regions generally display lower concentrations of microplastics than coastal areas, since most of these contaminants originate from the coast and river plumes and then are advected offshore by complex currents (Barnes et al. 2009). Similarly, our results revealed particular spatial patterns in particle distributions, with lower concentrations over the shelf, for different types of microplastics and biogenic particles (e.g., vascular plant detritus, macroalgae fragments, marine aggregates and exuviae).

The second most abundant group of particles were globose opaque particles. Still, their origin remains unclear. Unfortunately, the methodology used in this study did not permit the identification with certainty of the origin or typology of this group, which may also contain microplastics. Although opaque particles could be distinguished from PP&PE by the well defined contour of the latter and subtle differences in their grey levels, there is still some uncertainty regarding these particles. Thus, the real microplastics concentration could be even higher than reported in our data. Clearly, further studies are necessary to elucidate the origin of globose opaque particles in tropical coastal areas. This is the first attempt to use a laboratory-based semi-automatic optical method (i.e., the ZooScan approach) for this purpose.

6.4.3 Sources and sinks of microplastics

Microplastics may enter the seas from distinct land-based sources, from ships and other facilities at sea, from punctual and diffusive sources, and can travel long distances before they are stranded, degraded or buried in the sediment (Ryan et al., 2009).

Polyethylene and polypropylene are widely produced and used for many applications, and since approximately half the world's population resides within fifty miles of the coast, these materials have a high potential to reach the marine environment via rivers, wastewater systems, and by winds blowing offshore (Moore et al., 2001; Derraik, 2002; Thompson et al., 2005; Cole et al., 2011). In addition, polymers are highly persistent in the marine environment and their degradation is slow, even when exposed to strong UV radiation (Andrady 2003). The estimates on plastics longevity are highly variable but likely in the range of hundreds or even thousands of years, depending on the physical and chemical properties of the polymer (Teuten et al 2007; Galgani et al., 2015). This feature is one of the main reasons for the high concentration and ubiquity of these pollutants worldwide, including tropical coastal seas.

Total microplastics, polyethylene and polypropylene displayed higher concentrations in the Estuary and in the Bay, when compared to the Shelf, indicating a dilution gradient, sedimentation and degradation oceanward. These polymers have lower densities than seawater, so they float until they are washed ashore or even sink because their density changes due to biofouling and leaching of additives (Galgani et al., 2015). This coastal-offshore gradient in the PP&PE concentration is indicative of coastal sources and continuous particles degradation and sedimentation.

Several studies around the world showed high concentrations of polyethylene and polypropylene in offshore waters. The high concentration of these types of microplastics observed in the Bay area were also observed in other marine systems. Pabortsava and Lampitt (2020), on the surface of the Atlantic Ocean, showed that both inputs and stocks of ocean plastics are much higher than those determined since the 1950s, and polypropylene and polyethylene were responsible for the highest mass concentrations among the investigated microplastics. Another study by Kedzierski et al. (2019) in the Mediterranean Sea proposed a protocol to determine the microplastics concentrations necessary to provide representative data, and also concluded that polyethylene and polypropylene were the main polymers on the samples. Same results were found by Cincinele et al. (2017) in subsurface waters near-shore and off-shore the coastal area of the Ross Sea (Antarctica). These authors found predominantly polyethylene and polypropylene, among other types of microplastics, also using FTIR spectroscopy to determine particle typology.

On the other hand, the higher nylon concentrations over the mid-shelf indicate important nylon sources over the Shelf, e.g., derived from fisheries activities, and/or possibly a sink of nylon in the Bay area. Alternatively, the mid-shelf may receive inputs of large-scale estuarine plumes (e.g, from the nearby Una river), that may be rich in microplastics, PP & PE and nylon fibres.

6.4.4 Considering other particles and zooplankton during the evaluation of the impact of microplastics on marine food webs

Microplastics have been widely studied in recent years not only because of their increasing abundance in the water and in sediments, but also because they are being detected in numerous organisms throughout freshwater, estuarine and marine food webs (Browne et al., 2008; Collignon et al., 2012; Cole et al., 2013; Kaposi et al., 2014; Desforges et al., 2015). These microplastics can affect marine organisms whether chemically, due to the toxic effects of POPs (Persistent Organic Pollutants (Sobral et al., 2011) or physically, by damaging their digestive system (Cole et al., 2013). There are several laboratory studies that showed a variety of plankton organisms feeding on microplastics (Kremer and Madin, 1992; Cole et al., 2013; Botterell et al., 2019). These microplastics begin to accumulate in food webs (Setälä et al., 2014; Vandermeersch et al., 2015; Sun et al., 2017). There are few studies that assessed the effect of microplastic intake by zooplankton organisms on their feeding activity (Cole et al., 2013; Cole et al., 2015; Desforges et al., 2015; Sun et al., 2017). Cole et al. (2013) showed that the presence of microplastics drastically affects the ingestion of food items.

Yet, there are no published laboratory studies available that investigated the effect of the abundance of other food items (e.g., plankton and biogenic particles) on microplastic intake by zooplankton organisms. Numerous studies have shown that selective feeding by zooplankton on a given food item depends on the availability, abundance and exposure time of alternative food sources (Lenz, 1977; Paffenhöfer and Van Sant, 1985; Chong et al., 2001; Schwamborn et al., 2004; Schwamborn et al., 2006; Feehan et al., 2017).

Clearly, the presence of other food items available for zooplankton will affect their intake of microplastics, and thus the impact of microplastics on marine food webs. Therefore, it is essential to consider the abundance and composition of plankton and biogenic particles when assessing the impact of microplastics on coastal environments.

6.4.5 Relative microplastics concentration (RMC) as a key index of contamination

The rationale behind the proposed RMC (Relative Microplastics Concentration) is that the relative concentration is an index for the probability of encounter and ingestion of microplastics in the water column at any given location.

The use of relative indices, such as RMC, assumes that a given consumer will most likely ingest a given particle (including microplastics), depending on its availability in relation to food particles (Schwamborn et al., 2006). For example, even a low absolute concentration of microplastics may be extremely deleterious for planktivores and suspension feeders, if all other available food particles are scarce, too, as observed in the Shelf area in the present study. Conversely, in the Rio Formoso Estuary, microplastics showed extremely high concentrations and volumes, but other particles and zooplankton were even more abundant. Thus, RMC was low in the Estuary and consequently, the probability of microplastics being ingested was relatively low in the mangrove-lined Estuary, for total microplastics, PP & PE and nylon fibres. Conversely, extremely low concentrations of zooplankton and other particles were found in the reef-lined Bay and at nearshore shelf stations, contributing to the maximum RMC values found in these two areas.

For nylon fibers, an unexpected pattern was observed, where maximum RMC (i.e., maximum impact) was observed in offshore shelf areas, indicating that these offshore areas were most severely impacted by nylon fibres.

6.5 Conclusions

This study provided new insights into the distribution of microplastics within the available food spectrum in tropical coastal areas. The composition of biogenic particles followed the expected pattern, with more plant matter (mangrove detritus) in the mangrove-lined estuary. Surprisingly, the impact of microplastics was more severe in offshore waters than at the river mouths (the sources of anthropogenic particles). In short, Bay and nearshore shelf waters were the most severely impacted areas by total microplastics and PP & PE, and offshore waters with nylon fibres, although absolute concentrations of all microplastics, total particles and zooplankton were much higher in the estuary. This indicates that the impact of these persistent pollutants may be more harmful in oligotrophic offshore waters, than in particle-rich estuarine waters, opening a new line of research.

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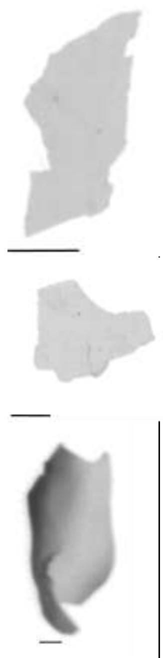
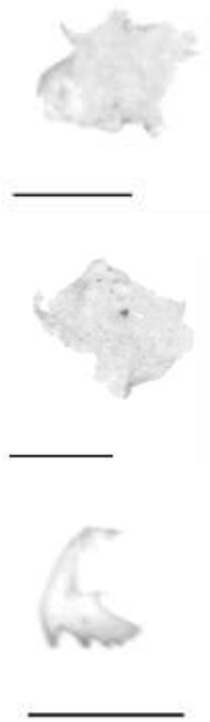
Supplementary materials

Suppl. Table 1. Descriptive statistics of the environmental variables (Temperature, Salinity and Secchi depth) during the dry and rainy seasons. Bimonthly sampling was conducted form 2013 to 2015 in the Rio Formoso Estuary, Tamandaré Bay and on the Continental Shelf. Asterisks represent significant differences between rainy and dry season, for Secchi depth.

					Dry				
		Estuary			**Bay**			**Shelf**	
	min	*mean±std*	*max*	*min*	*mean±std*	*max*	*min*	*mean±std*	*max*
Temperature	27.29	28.34±0.71	29.50	27.56	28.67±0.71	29.52	27.07	27.85±1	28.74
Salinity	27.94	32.8±2.19	35.92	35.58	36.085±0.39	36.54	33.78	36.46±1	36.73
Secchi disk	1.20	1.5±0.29	2.00	2.30	3.5±1.16 *	5.50	2.50	5±4	19.00
					Rainy				
Temperature	26.70	28.41±1.28	30.54	27.00	28.81±1.12	30.28	26.38	28.315±1	29.76
Salinity	27.03	31.19±2.92	36.43	35.00	35.68±0.47	36.54	34.67	36.18±1	37.44
Secchi disk	1.50	2±0.51	3.50	1.50	3±0.91	4.50	1.00	6±4	14.00

Suppl. Table 2. according to ZooScan and FTIR classifications.

1	2	3	4	5	6
					
Polymers PP & PE	*Nylon (Polyamide)*	*Plant detritus*	*Marine aggregates*	*Macroalgae*	*Exuviae*
Different shades of gray but Well-defined shape and ends	Nylon fibers	Vascular plant fragments such as leaves, roots or twigs	Gelatinous or mucous structures that can aggregate organisms and particles in their structure	Macroalgae fragments	Exuviae of small crustaceans (mostly copepods)and adult barnacles

7	8	9	10	11	12
					
Sand	Opaque	Transparent	Dark flat	Opaque flat	Transparent flat
Sand Grains	Unknown origin*. Show irregular, three-dimensional or globose shape and homogeneous, dark grey levels.	Unknown origin*. Show irregular, three-dimensional or globose shape and light grey levels.	Unknown origin*. Flat shape, black. Ink or paint fragments?	Unknown origin*. Flat shape, dark grey.	Unknown origin*. Flat shape, light grey.

*: It was not possible to relate these vignettes to the identifications using FTIR.

**: scale bar: 1mm

Suppl. Table 3: Particles concentration (counts m³), according to location, and season. Bimonthly samples from 2013 to 2015 in the Rio Formoso Estuary, Tamandaré Bay and on the Continental Shelf.

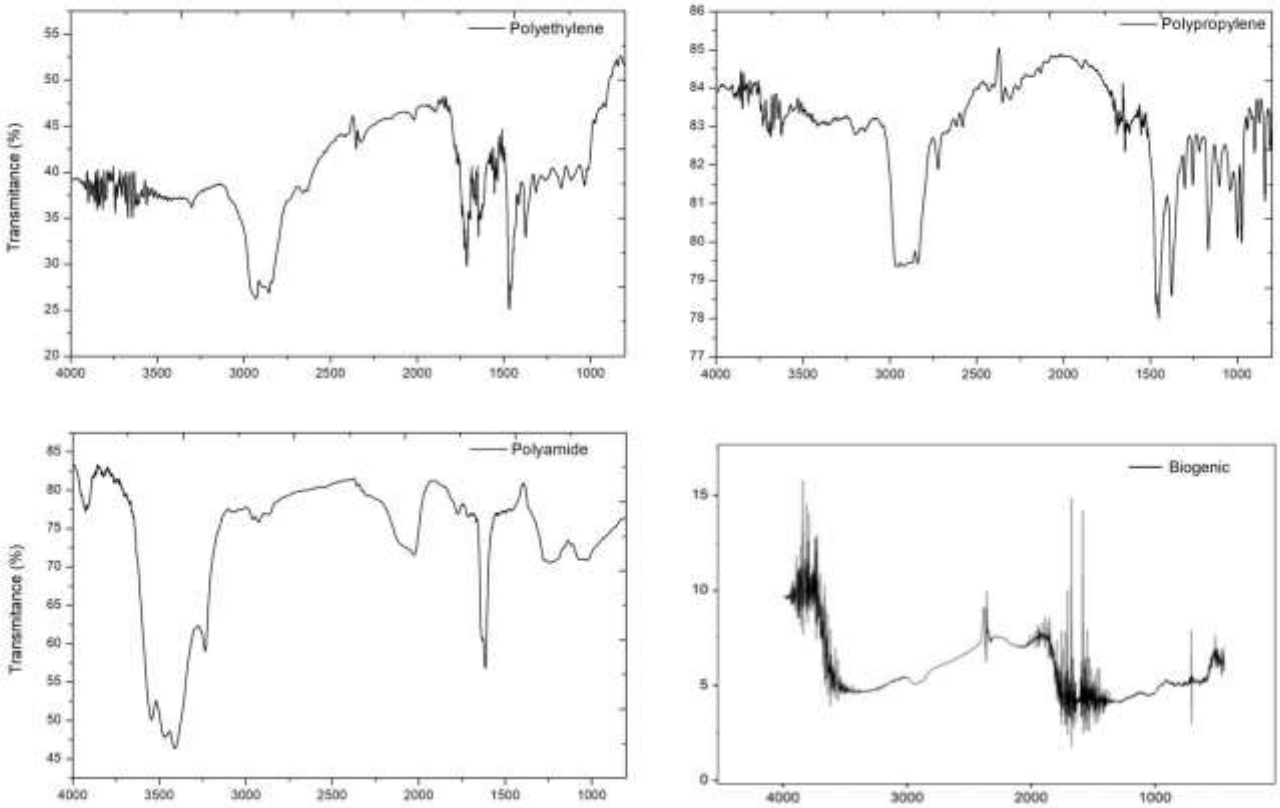
	Particles concentration (counts m^3)																	
	Dry									**Rainy**								
	Estuary			**Bay**			**Shelf**			**Estuary**			**Bay**			**Shelf**		
	Min	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**	**Average**	**Max**
PP & PE	0.84	24.31	117.99	0.38	3.74	20.46	0.04	1.82	16.24	1.38	27.98	267.96	0.08	1.72	6.36	0.05	1.23	12.69
Nylon	0.80	36.83	103.27	0.52	8.38	103.49	0.24	1.83	12.61	0.95	41.20	338.56	0.62	2.96	6.36	0.05	1.17	7.27
Plant origin	4.67	148.08	457.20	2.47	18.90	142.56	1.07	9.06	66.01	3.22	214.80	1802.30	2.41	8.34	15.33	0.31	6.16	32.63
Marine aggregates	5.01	142.24	462.49	2.80	20.93	143.11	0.94	6.94	45.11	3.56	197.44	1280.80	2.10	8.57	20.43	0.26	5.23	29.07
Macroalgae	0.02	2.21	22.20	0.01	0.40	2.94	0.01	0.13	0.46	0.01	3.35	35.73	0.02	0.09	0.49	0.01	0.13	0.59
Exuviae	1.06	17.95	53.95	0.03	1.25	7.33	0.01	0.56	2.67	0.26	24.72	170.54	0.04	1.10	3.93	0.01	0.61	2.42
Sand	0.02	9.20	35.97	0.01	0.47	3.38	0.01	0.69	7.09	0.01	8.92	73.47	0.28	0.17	0.72	0.01	0.47	3.63
Opaque	3.09	70.54	235.98	0.72	8.76	58.64	0.35	5.01	41.33	1.75	88.69	587.15	0.85	4.64	10.92	0.15	2.83	14.61
Opaque flat	0.02	6.70	35.54	0.01	0.13	0.64	0.01	0.18	0.95	0.02	8.25	64.83	0.07	0.41	1.57	0.01	0.17	0.65
Dark flat	0.01	2.90	14.75	0.02	0.54	2.92	0.01	0.21	1.99	0.05	10.94	70.59	0.01	0.32	1.57	0.01	0.40	5.40
Transparent	0.67	26.68	88.49	0.43	4.42	38.81	0.23	1.30	9.57	0.69	17.65	146.98	0.42	2.02	5.32	0.08	0.89	3.67
Transparent flat	0.01	0.35	4.08	0.01	0.15	1.25	0.01	0.10	2.30	1.52	5.03	64.83	0.01	0.14	0.79	0.006	0.14	1.80
Total	16.22	487.99	1631.91	7.41	68.07	525.53	2.93	27.83	206.33	13.42	648.97	4903.74	6.91	30.48	73.79	0.946	19.43	114.43

Suppl. Table 4. Permutation Test (PERMANOVA) for abundance and relative abundance.

Pairwise post-hoc comparisons: Estuary vs Bay, Bay vs Shelf and Estuary vs Shelf.

Particle type	*Estuary vs Bay*	*Bay vs Shelf*	*Estuary vs_Shelf*
Concentration (counts m³)			
PP & PE	0.156564095	< 0.0001	<0.0001
Nylon	0.062576304	0.329046703	0.619637259
Plant derived	5.04E-09	0.409397953	< 0.0001
Marine aggregates	0.700500979	0.34423789	0.067789837
Macroalgae	0.536873757	< 0.0001	0.000393121
Exuviae	0.06548533	0.01083509	< 0.0001
Sand	0.162813715	0.804504103	0.41167328
Opaque	0.046541626	< 0.0001	< 0.0001
Transparent	0.251279219	< 0.0001	< 0.0001
Dark flat	0.885953905	< 0.0001	< 0.0001
Opaque flat	0.620716462	< 0.0001	< 0.0001
Transparent flat	0.468318566	0.00021673	< 0.0001
Total particles	< 0.0001	0.008776125	< 0.0001
Volume (mm^3 m^{-3})			
PP & PE	0.478131262	0.01941565	0.000309154
Nylon	0.17913744	0.063782484	0.921413771
Plant derived	0.006240812	0.561585027	0.076534332
Marine aggregates	0.928420156	0.001406116	0.000353139
Macroalgae	0.182401556	< 0.0001	0.003122955
Exuviae	0.615432744	0.999992292	0.588786936
Sand	0.194028677	0.935987171	0.081131335
Opaque	0.05314817	0.050715715	< 0.0001
Transparent	0.676084897	< 0.0001	< 0.0001
Dark flat	0.888975273	< 0.0001	< 0.0001
Opaque flat	0.744655908	< 0.0001	< 0.0001
Transparent flat	0.524672154	0.000162744	< 0.0001
Total particles	0.000346872	0.067670406	0.168768653
Concentration (%)			
PP & PE	< 0.0001	< 0.0001	0.525202602
Nylon	0.360345466	0.084355557	0.001205349
Plant derived	< 0.0001	0.43012397	0.000329077
Marine aggregates	0.001730781	0.101029206	< 0.0001
Macroalgae	0.001039043	< 0.0001	0.087650681

Exuviae	< 0.0001	0.498576975	0.000206918
Sand	0.016719387	0.926243626	0.034563716
Opaque	0.377733499	0.000195505	0.027076469
Transparent	0.067141722	< 0.0001	< 0.0001
Dark flat	0.968313284	< 0.0001	< 0.0001
Opaque flat	0.480876188	< 0.0001	< 0.0001
Transparent flat	0.780973206	< 0.0001	< 0.0001
Volume (%)			
PP & PE	0.346402365	0.004766099	0.215239522
Nylon	0.927369411	0.470905694	0.269078828
Plant derived	0.516490844	0.939248597	0.699327602
Marine aggregates	0.047989413	0.070567431	< 0.0001
Macroalgae	0.057046271	< 0.0001	0.010415674
Exuviae	0.000392835	0.123680626	0.103943489
Sand	0.092752912	0.993736566	0.097246508
Opaque	0.396820653	0.074580039	0.001281811
Transparent	0.817816114	< 0.0001	< 0.0001
Dark flat	0.984198414	< 0.0001	< 0.0001
Opaque flat	0.960353976	< 0.0001	< 0.0001
Transparent flat	0.904993249	< 0.0001	< 0.0001



Suppl. Fig.1. FTIR (Fourier-transform infrared spectroscopy) spectra of biogenic particles, polyethylene, polypropylene, and polyamide (i.e., nylon).

7 ARTIGO 3: WHAT DETERMINES THE SHAPE OF THE COMPLETE SIZE SPECTRUM (INCLUDING PLANKTON, MICROPLASTICS AND OTHER PARTICLES) IN TROPICAL COASTAL ECOSYSTEMS?

Estado: A ser submetido - Progress In Oceanography

ABSTRACT

Size-spectra analysis has a solid theoretical and empirical foundation and can be used to provide useful information on ecosystem structure. The objective of this study is to obtain complete Normalized Biovolume Size Spectra (NBSS), including zooplankton, microplastics, and other suspended particles, along an estuary-shelf gradient. Plankton net samples (300 micrometer mesh) were obtained in the Rio Formoso Estuary, in Tamandaré Bay and on a transect across the continental shelf off Tamandaré, Brazil, in two years (from April/2013 to May/ 2015) at bi-monthly intervals, totalizing 112 samples. Particles were identified by image analysis (ZooScan) and infrared spectroscopy (FTIR). Generally, NBSS slopes were close to -1 (i.e., between -1.09 and -0.85), except for NBSSz (zooplankton only) in the Estuary (-1.59) and in the Bay (-1.44), where the steepest slopes were observed, due to the importance of small-sized zooplankton in these areas. The NBSSz slope was significantly steeper in the Estuary and in the Bay than on the Shelf. The inclusion of particles into the NBSS (NBSSp) turned the slope significantly less steep in the Estuary and in the Bay. In spite of parallel slopes, intercepts were significantly higher in the Estuary than in the other two areas, after including particles in the analysis (NBSSp), due to the extremely high total volume of biogenic particles in the estuary, and the parallel shapes of the NBSS for biogenic particles in the three sampling areas. The most relevant impacts of microplastics were detected within the larger size classes (> 6.5 $\log_{10}$ μm^3). In the Estuary, large-sized microplastics were similarly important (in terms of volume) as zooplankton, in both seasons. Large-sized PP&PE were more relevant in the Bay (in both seasons), and large-sized nylon fibres on the Shelf (in the rainy season). The present study constitutes

a first, pioneering effort for a synthetic analysis of zooplankton, microplastics and other particles, inaugurating a new line of size-spectra-based research in marine ecosystems.

Keywords: NBSS; Size structure; ZooScan; FTIR; Spatial variability; Tropical South Atlantic; Mangrove; Reef ecisystems.

7.1 Introduction

Size-based indices have a solid theoretical and empirical foundation and can be used to describe plankton communities (Sheldon et al., 1972; Gaedke, 1992; Jennings et al., 2001; Zhou et al, 2009; Marcolin et al., 2013; Dai et al., 2016), to assess productivity, energy transfer efficiency and as a proxy for environmental status, providing useful information on ecosystem structure (Cohen et al., 1993; Shin et al., 2005; Zhou, 2006). The NBSS (Normalized Biomass Size Spectrum) theory takes into account the size spectra of a community (Sheldon et al. 1972; Zhou 2006), based on the rationale that larger organisms feed on small ones (Shin et al., 2005; Blanchard et al., 2009).

The NBSS is calculated by allocating organisms into size classes according to their individual body size and then computing the total mass in each size class (Gaedke, 1992; Zhou et al., 2006). This accumulated biomass is normalized by the weight of the size class (measured in size, volume or mass). This distribution of mass over size is usually represented by plotting the size classes in the x-axis and the normalized mass in the y-axis, both in log scale (Herman and Harvey, 2006). Several studies have shown that the NBSS shapes calculated for pelagic communities, will generally produce perfectly linear shapes, and that the slope of the line fitted to the NBSS can be used to compare ecosystems and to assess food web features , such as energy transfer efficiency (Figueiredo et al., 2020). Theoretically, in a stable marine ecosystem, the mean slope of NBSS is approximately −1 (Sprules and Munawar 1986). Steeper NBSS slopes have been associated with lower trophic efficiency and higher contributions of small organisms to the total biomass (Sprules and Munawar 1986; García-Comas et al., 2014; Sato et al., 2015).

One convenient way to obtain zooplankton size spectra is the ZooScan approach, a bench system that allows to semi-automatically evaluate preserved plankton samples, based on scanned images that provides estimates of size, volume and other metrics

(Grosjean et al., 2004; Gorsky et al., 2010, Forest et al., 2012; Marcolin et al., 2013; García-Comas et al., 2014). This method was developed with a focus on the zooplankton, but it can also be used to identify microplastics and other particles (Lins Silva et al., 2019; Lins-Silva et al., 2021). In addition, it is possible to combine other equipments, such as the Reflectance Infra-Red Spectroscopy (FTIR - Fourier-Transformed Infrared Spectroscopy), to analyse and distinguish particle types, and improve classification of particles, such as nylon and polyethylene-polypropylene particles (Lins-Silva et al. 2021), and finally provide size spectra analysis for microplastics.

In spite of the recent and fast increase of studies focusing on size spectra, most ZooScan studies treat non-living particles as noise that needs to be removed from the analysis (Marcolin et al., 2013; García-Comas et al., 2014; Figueiredo et al., 2020). Previous studies (Lins Silva et al., 2019; Lins-Silva et al., 2021) have shown that non-living particles (e.g., marine aggregates, plant detritus, microplastics, etc.) may represent up to 50% in volume of plankton net samples.

The NBSS slopes and intercept of the spectrum including zooplankton and particles, as proposed in this study, may serve as a synthetic comparison with the sestonsize spectrum, and allow a full description of the available food spectrum for planktivores in pelagic ecosystems. Here, we analyze under which circumstances the size spectrum of zooplankton is more impacted by microplastics and other suspended particles, (i.e, how the spectrum changes when such particles are included). We used this approach to test the hypothesis that the inclusion of particles into the analysis will significantly affect the results of size spectra assessments in coastal ecosystems.

7.2 Methods

7.2.1 Study area

Samples were collected around three marine protected areas: Rio Formoso Estuary, Tamandaré Bay and the Continental Shelf off Pernambuco (State Decree, nº 19.635, March 13, 1997 and Federal Decree, s/n, October 23, 1997; CPRH 1998). The Rio Formoso Estuary (8° 39' - 8° 42′S and 35°10' - 35° 05′W), area highly turbid, “brown” estuarine waters, covered by mangroves with muddy sediments rich in organic matter, which seem to be the most important source of suspended matter in the estuarine area (Silva, et al., 2003; Vasconcelos et al., 2004). Tamandaré Bay (8° 44' - 8° 47′ S and 30° 0.5' - 35.07′ W) located ~4 km South of the Rio Formoso Estuary, is a coastal embayment lined by several parallel sandstone reefs with high sedimentation rates and low hydrodynamics, which promote water retention (Rebouças, 1966; Camargo et al., 2007). Marine erosive processes observed in this area are related to anthropic interventions associated with irregular urban expansion (CPRH, 1999). And Continental Shelf off Pernambuco, an area relatively narrow (~35 km wide), with shallow depths. The adjacent inner shelf of Tamandaré has a linear sandstone rocky beach parallel to the coast, which is a substrate for algae and coral growth, and also an effective protection against wave energy. The water above the shelf break has 50 meters, high salinity and warm water (Manso et al., 2003; Camargo et al., 2007).

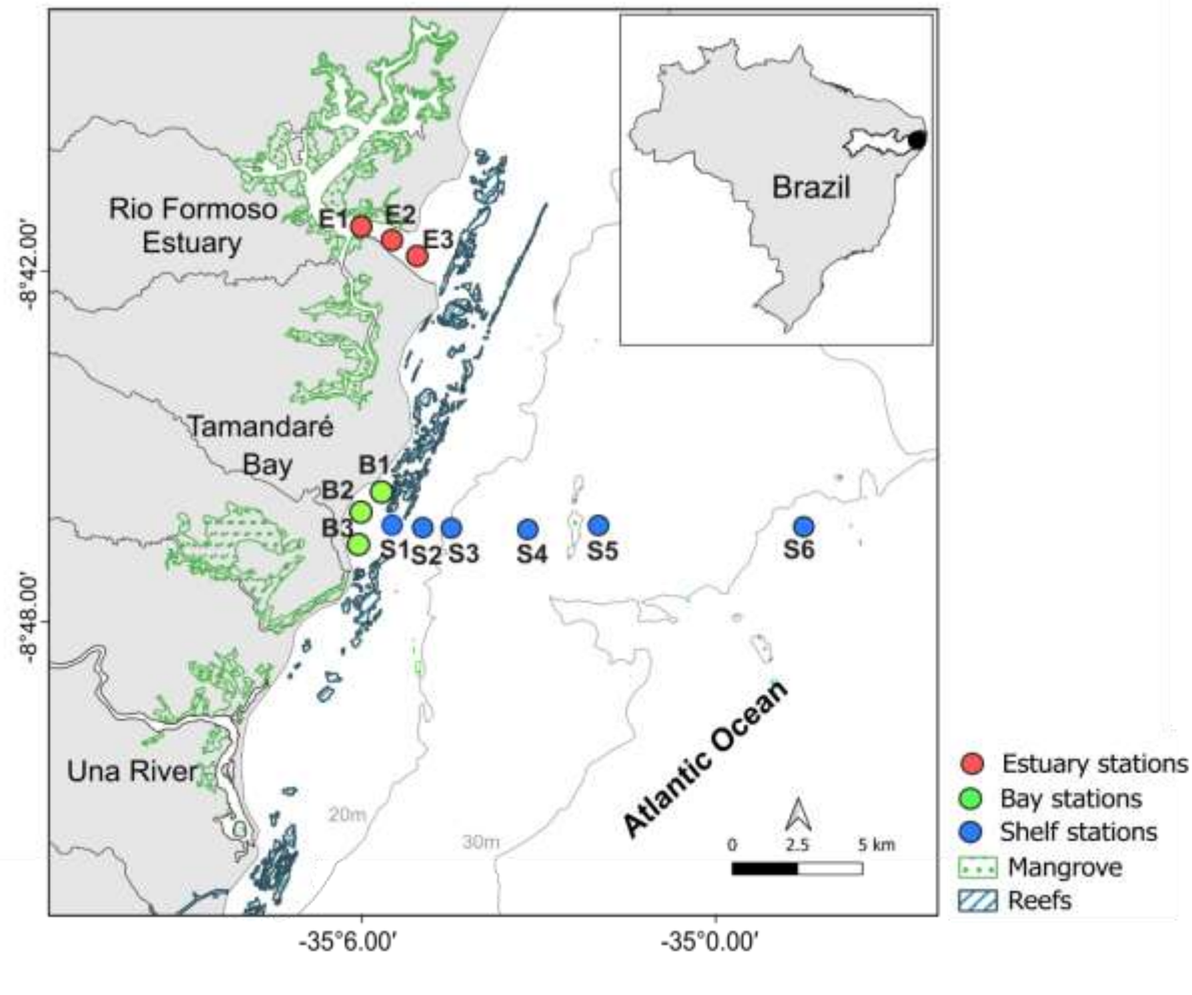


Fig. 1. Map of the Tropical Atlantic Ocean showing the coast of Brazil and location of sampling stations off north-eastern Brazil, in April 2013 and May 2015. Color codes

indicate sampling stations at Rio Formoso Estuary (yellow circles), Tamandaré Bay (orange circles) and on the Continental Shelf (blue circles).

7.2.2. Plankton sampling and hydrological parameters

Three fixed stations were established in the Estuary and in the Bay, and six stations on Shelf (three stations at the nearshore shelf and three at mid-shelf), (Fig. 1) during daytime (05:00 AM to 04:59 PM) between April 2013 and May 2015. At each station, horizontal hauls were performed at the subsurface using a conical plankton net (300 µm mesh size, 0.6 m mouth opening diameter and 2.5 m net length). A flowmeter (Hydro-Bios, Kiel, Germany) was used to estimate the filtered volume. All samples were preserved with 4% formaldehyde, buffered with sodium tetraborate (0.5 g L-1). A total of 112 plankton samples were analyzed. in the project context ESPLAN and INCT AmbTropic. At each station, salinity and temperature data were measured using a CTD probe (CastAway, YSI). Water transparency was estimated using a Secchi disk (Preisendorfer, 1986).

7.2.3. Image acquisition and analysis by ZooScan and FTIR - Fourier-Transformed Infrared Spectroscopy analysis

Abundance and biovolume of zooplankton and particles using digital images obtained with the semi-automatic ZooScan system (Hydroptic model ZSCAN03) and its associated software, the ZooProcess (http://www.obs-vlfr.fr/LOV/ZooPart/ZooScan).

Samples were separated into two size fractions (>1000µm and < 1000µm) to avoid underestimating large organisms and particles (Gorsky et al., 2010) with 2400 dpi resolution, as established by Grosjean et al. (2004; http://www.zooscan.obs-vlfr.fr/). Samples obtained from estuarine and Bay areas were diluted in filtered water, according to the concentration of particles, and then carefully mixed before the extraction of 10 ml fraction. On the other hand, samples from continental shelf were separate in aliquots (1/4 to 1/128) using a Motoda splitter (Motoda, 1959), and put it into the ZooScan, for manual separation of the organisms, for no longer than 20 min per sample, to avoid images with overlapping organisms and/or particles (Gorsky et al., 2010; Vandromme et al., 2012).

ZooProcess (Version 7.25), and ImageJ (https://imagej.nih.gov/ij/) software were used to process images and vignettes. The lower recognition size limit of particles was set to 300 µm reported equivalent spherical diameter (ESD). Plankton Identifier (PkID) software (version 1.3.4), was used for semi-automatic classification. First, we built a training set, which was used in Random Forest, an algorithm for the automatic classification of vignettes into predefined categories. After the classification, all vignettes were manually validated to correct any misclassification. The validated results tables were processed to remove bubbles, noise, scanning artifacts, and vignettes with multiple objects. Size parameters were converted from pixels to micrometers, according to the scanner resolution (size of 1 pixel at 2400 dpi: 10.58 µm). To minimize possible sample contamination with microplastics on board and in the laboratory, we applied a series of protocols and blanks, e.g., we used filtered water (20 micrometer mesh filters), and all sample flasks and materials were thoroughly washed before placing the sample. Zooplankton was classified based on the ZooScan vignettes, according to the relevant taxonomic literature. All non-zooplankton particles were separated and classified by a combination of elutriation, FTIR (Fourier-Transformed Infrared Spectroscopy analysis) and ZooScan methods (Lins-Silva et al., 2021). Biogenic particles were separated from microplastics according to their density (elutriation) in solvents of different polarity and density (Lins-Silva et al., 2021). All FTIR spectra were treated using the OriginPro 8 software. The identification of polymers was made by comparing the sample spectra with standard spectra contained in the Hummel Polymer and Additives library (Silverstein, et al., 2007).

7.2.4 Zooplankton and particles volumes

Ellipsoid volume (mm^3) was generally estimated based on the major and minor axes of the equivalent ellipse, obtained from ZooScan vignettes (Vandromme et al., 2012, Stemmann and Boss, 2012). Flat particles and fish larvae were considered as having a flat shape and their volume (mm^3) was calculated based on the surface area (the“area_exc” parameter; mm^2) multiplied by the thickness of each particle type (Grosjean et al., 2004). Thickness was measured under a stereo microscope (Zeiss, Stemi SV6 model) in 30 randomly chosen plankton samples as described by Lins Silva et al., 2019.

7.2.5 Normalized biovolume size spectra (NBSS)

Normalized biomass size spectra (NBSS, Platt and Denman, 1977, 1978) were calculated based on the ellipsoid biovolumes obtained from ZooScan (Vandromme et al., 2012). First, the total particle biovolume (mm³) per cubic meter of filtered water (mm³ m^{-3}) was classified in geometrically increasing biovolume size classes (mm³). Normalized biovolume (m^{-3}) was obtained by dividing the total biovolume (mm³ m^{-3}) value in each size class by the respective biovolume size class width (mm³). NBSS slopes and intercepts were calculated by fitting a linear regression to each spectrum using log-transformed data. Previous to regression analysis, the smaller (not efficiently sampled) and large size classes (rare large organisms and particles, with many empty bins) of each spectrum were removed to compensate for the low sampling efficiency of the net at the extremes of the spectrum (i.e., mesh selectivity and gear avoidance). Thus, we only included data between the peak of the distribution (maximum) and the first empty size class in our analyses. We calculated three types of NBSS: for zooplankton only (“$NBSS_Z$”), for all scanned particles (zooplankton and other particles, including microplastics, “$NBSS_P$”), and for zooplankton and other particles, excluding microplastics (“$NBSS_N$”).

7.2.6 Statistical analyses

Two-way PERMANOVA (Anderson, 2001) was used to test for differences in slopes and intercepts according to three factors: i) “seasons” (dry *vs* rainy), ii) “areas” (Estuary, Bay and Shelf) and iii) their interaction (seasons *vs* areas). PERMANOVA was conducted (20,000 permutations), based on the euclidean distance (Anderson, 2017), applying the function “adonis” within the “vegan” R package (Oksanen et al., 2017). For for the factor “areas” (three categories), post-hoc tests for pairwise multiple comparisons of mean rank sums (i.e., non-parametric Kruskal-Nemenyi tests, Nemenyi, 1963), were conducted when PERMANOVA indicated significant effects ($p < 0.05$).

One-way Permanova and Nemenyi post-hoc pairwise tests were also used for comparing NBSS parameters (slopes and intercepts) of zooplankton only (“NBSSz”), zooplankton and particles, including microplastics (“NBSSp”), and zooplankton and particles, without microplastics (“NBSSn”).

7.3. Results

7.3.1 Zooplankton composition and spatial distribution

Twenty-six zooplankton taxa were identified. In terms of numerical abundance, four important zooplankton groups were found: copepods of the orders Calanoida and Cyclopoida, followed by decapods (e.g., zoea- and megalopa-stage larvae of brachyuran crabs and caridean shrimp zoeae) and chaetognaths. Calanoids were the most abundant group in all environments (Estuary, Bay and Shelf). In the Estuary, following calanoids, decapods were also very important in terms of relative abundance, decreasing oceanward. In the Bay, following Calanoida and Cyclopoida, bryozoan larvae had surprisingly high abundances in August (peak rainy season). On the Shelf, chaetognaths also had a high relative abundance, together with copepods (Calanoida and Cyclopoida) and decapods (Fig. 2a).

Copepods of the order Calanoida also presented the largest relative biovolume in all environments. There was a large contribution, in units of biovolume, of large-sized decapods, especially in the Bay, compared to their small relative abundance. Still in the Bay, in units of biovolume, small-sized bryozoan larvae were not representative, conversely to the large contribution of chaetognaths to the biovolume in the Bay.

On the Shelf, as well as in the estuarine and Bay areas, cyclopoid copepods were less important in units of biovolume. However, large-sized amphipods emerged as being an important group in units of biovolume on the Shelf, particularly in August (Fig. 2).

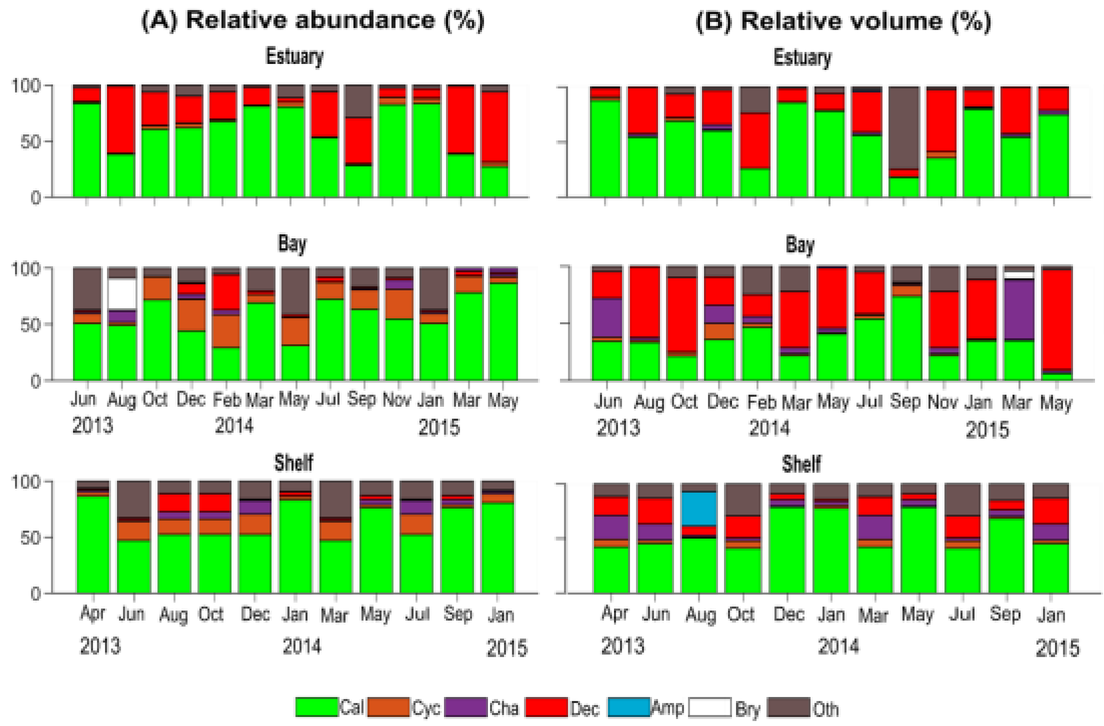


Fig. 2. Relative abundance and relative biovolume of the most abundant zooplankton taxa in three areas (Rio Formoso Estuary, Tamandaré Bay and Continental Shelf) off Tamandaré, Brazil, n = 112. Cal: Calanoida copepods; Cyc: Cyclopoida copepods; Cha: Chaetognaths; Dec: Decapods; Amp: Amphipods; Bry: Bryozan larvae; Oth: Others groups.

7.3.2 Size spectra shapes of zooplankton, microplastics and other particles

The contributions of zooplankton, microplastics and other suspended particles to the NBSS showed characteristic patterns for each type of particle and for each area (Fig. 3). In general, all NBSS exhibited the expected shape, with higher total volumes in the smaller size ranges and a log-linear decline towards larger size classes, for zooplankton, microplastics, and other particles.

Twelve types of non-organismic particles (two types of microplastics, eight types of biogenic particles, sand grains, and globose opaque particles) were categorized, counted and measured, using combined ZooScan+ FTIR analysis. In the Estuary, particles were mostly represented by biogenic particles (e.g., plant-derived detritus) and were the most important suspended solid objects (in total abundant and total biovolume) in the water column. The second most abundant group of particles were unidentified “globose opaque particles”. In the Bay, total biovolume of zooplankton and particles was much lower than in the Estuary. Following the estuarine-offshore gradient, the Shelf had the lowest overall total abundance and total biovolume of particles (mostly composed of marine aggregates) and zooplankton (Fig. 3).

The shape of the spectrum of zooplankton and that of “other particles” (mostly biogenic particles) was generally similar (Fig. 3), except that the first main peak for “other particles” was always found at smaller size classes than for zooplankton, in all areas. The first main peak of biogenic particles was located at approx. 4.5 $\log_{10}$ mm^3 (green lines in Fig 3), while it was at approx. 5.5 $\log_{10}$ mm^3 for zooplankton (black lines in Fig. 3), in all areas.

The volume ratio zooplankton / biogenic particles changed with particle size, especially in the Bay and in the Estuary. The change of the zooplankton / particles volume ratio with size was different between areas (Fig. 3). A parallel pattern was observed on the Shelf (i.e., there were parallel spectra for zooplankton and particles). Thus, on the Shelf, zooplankton was consistently more abundant than particles (i.e., when considering all size classes within the useful linear size range for zooplankton, from the zooplankton main peak to the first empty bins).

Conversely, a non-parallel “crossed” pattern was found in the Estuary and Bay areas (i.e., spectra were not parallel, the NBSS being conspicuously steeper for zooplankton than for particles). In these areas (Estuary and Bay), there was a striking difference between zooplankton (black lines in Fig. 3) and “other particles” (green lines in Fig 3): zooplankton was only more abundant than particles at the zooplankton main peak (approx. 5.5 $\log_{10}$ mm^3), while these particles were considerably more abundant than zooplankton (in the Estuary), or similarly abundant (in the Bay) in the subsequent larger size classes (within the useful linear size range).

The impact of microplastics (red and blue lines in Fig. 3) was particularly relevant within the larger size classes (> 6.5 $\log_{10}$ mm^3). In the Rio Formoso Estuary, microplastics were similarly important (in terms of volume) as zooplankton (in both seasons), within the size classes > 6.5 $\log_{10}$ mm^3. However, microplastics were always considerably less abundant than other particles in the Estuary, where extremely high abundances and total volumes of other particles were found, for all size classes.

In Tamandaré Bay, beads of PE & PE were especially abundant in the larger size classes (approx. 7 to 8 $\log_{10}$ mm^3). In this size range, beads of PP & PE were the most important suspended solid objects (in units of volume) in the water column, being more important than zooplankton and even more important than any other particles. This constitutes a very relevant impact for pelagic food webs, and was observed consistently in the Bay, for the dry and in the rainy season.

PP & PE usually had a higher total volume than nylon, throughout all size classes and in all areas, except at the Shelf during the rainy season, when large-sized nylon fibres dominated the larger volume classes (approx. 7 to 8 $\log_{10}$ mm^3). At the Shelf, during the rainy period, large-sized nylon fibres were more important (in units of total volume) than beads of PP&PE, and even more important than the other particles, within the larger size classes.

Thus, the impact of microplastics was particularly relevant within larger size classes (Fig. 3), with large-sized beads of PP&PE being more relevant in the Bay (for both seasons), and large-sized nylon fibres on the Shelf (in the rainy season).

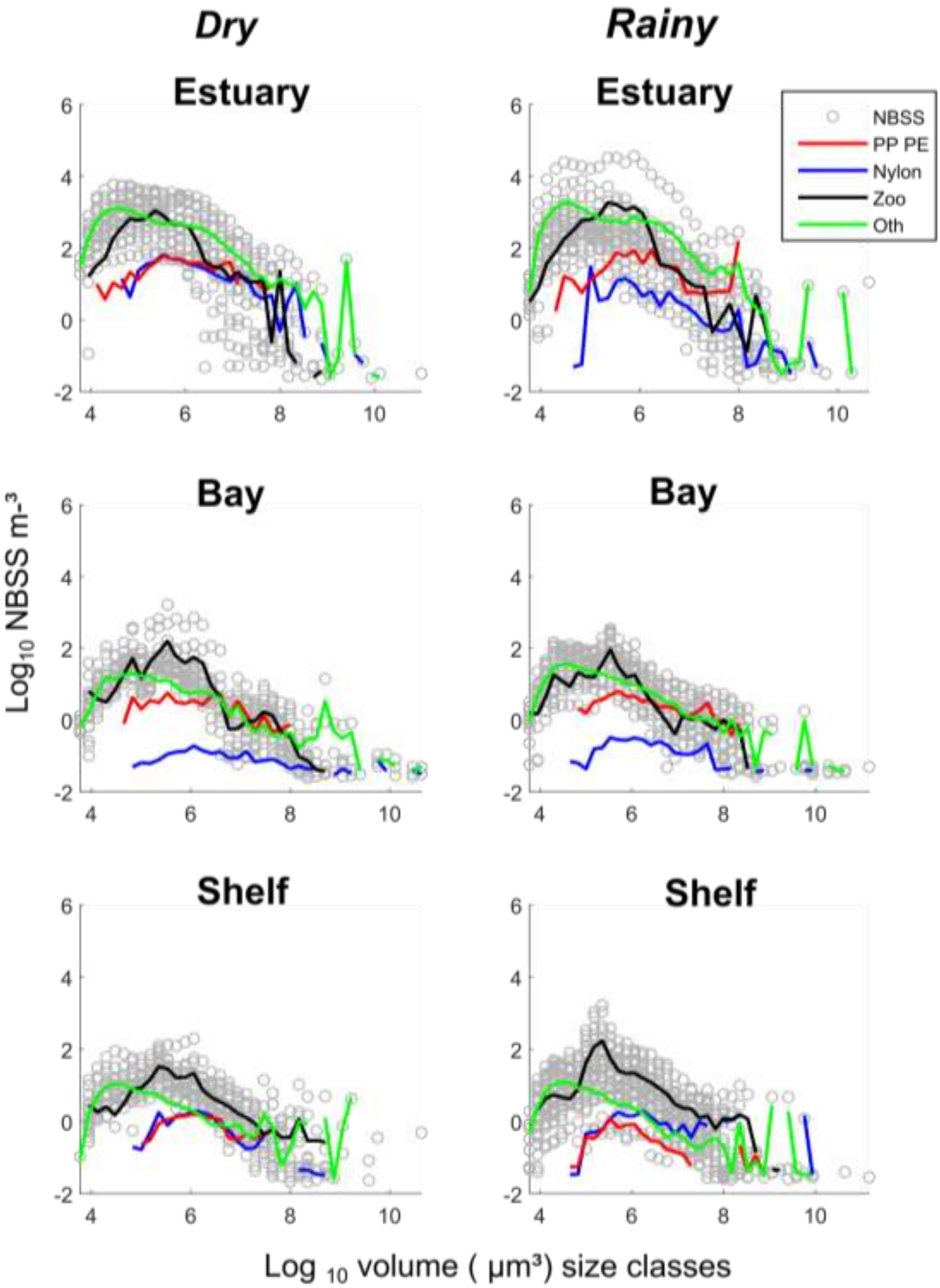


Fig. 3. Complete Normalized Biomass Size Spectra (NBSS, grey dots) obtained for each station and sample (sum of all particles + zooplankton), superimposed on the average NBSS for selected categories of particles (colored lines). NBSS: Normalized Biomass Size Spectra; PP PE: Polypropylene / Polyethylene; Nylon: Polyamide (nylon

fibers); Zoo: Zooplankton; Oth: All other suspended particles found in plankton samples, especially biogenic particles (e.g., plant-derived detritus, macroalgae fragments, marine aggregates and exuviae).

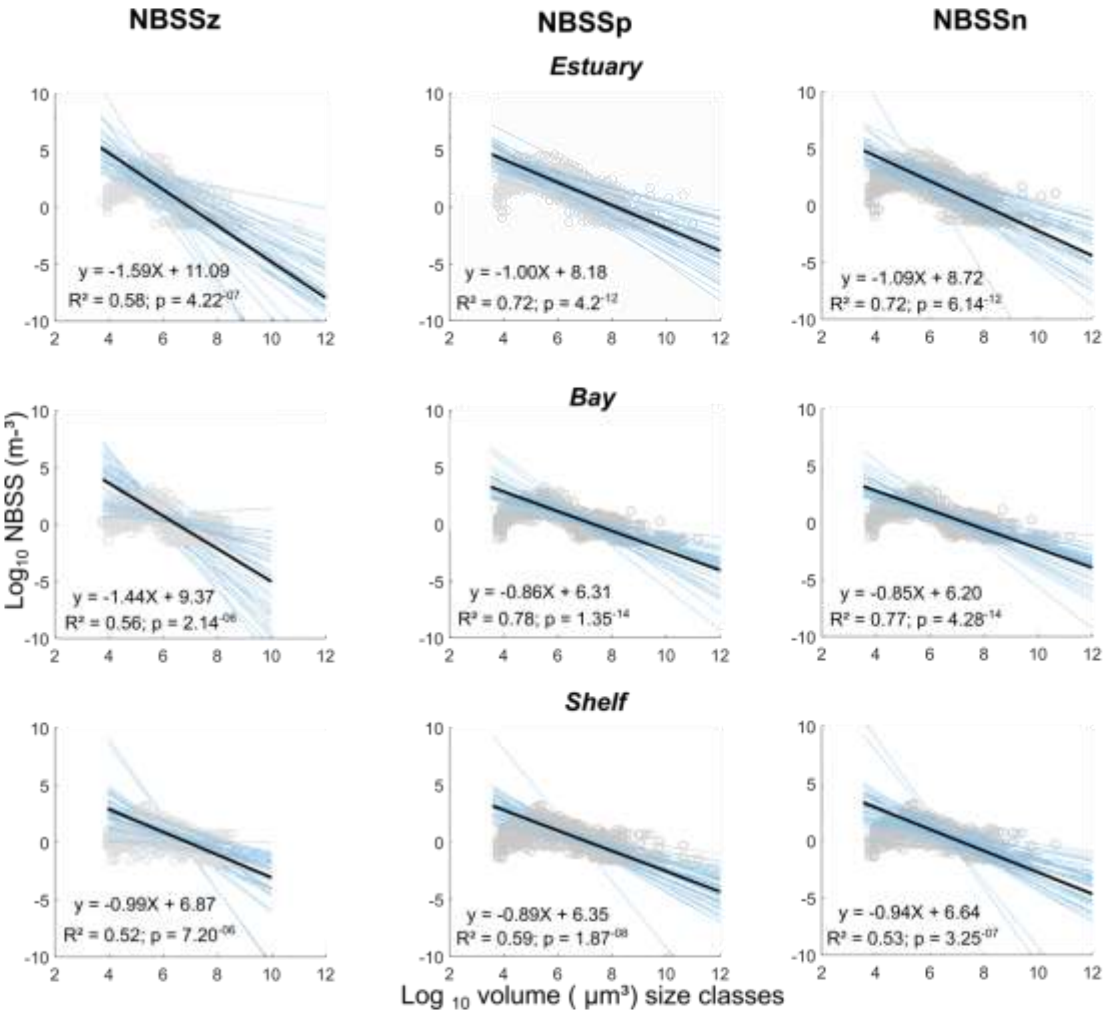


Fig. 4. Normalized Biomass Size Spectra (NBSS), comparing NBBS of zooplankton only (NBSSz), zooplankton and particles, including microplastics (NBSSp), and zooplankton and particles, without microplastics (NBSSn) in three sampling areas (Estuary, Bay and Shelf). Each blue line represents a NBSS for one station; the thick black line represents the mean NBSS for each dataset.

7.3.3 Comparing NBSS slopes and intercepts between areas and seasons

NBSS slopes and intercepts showed a high variability between stations, but characteristic and consistent differences between areas (Figures 4 and 5). Generally, mean NBSS slopes were close to -1 (i.e., between -1.09 and -0.85), except for NBSSz (zooplankton only) in the Estuary (-1.59) and in the Bay (-1.44), where the steepest slopes were observed (Fig. 4).

There were highly significant differences in slopes and intercepts between sampling areas (PERMANOVA, Table 1). However, there were no significant differences in slopes and intercepts between seasons (PERMANOVA, dry *vs* rainy), and there were no significant interactions between the factors "areas" and "seasons" (Two-way PERMANOVA).

Pairwise comparisons (Nemenyi post-hoc test, Table 1) considering analyses of zooplankton only (NBSSz) showed that Estuarine and Bay stations had significantly higher intercepts ($p < 0.001$) than the Shelf (Table 1, left graphs in Figure 5), mainly due to the fact that the Estuary and the Bay had more small-sized zooplankton volume than the Shelf (Figure 3). The high total biovolume of small-sized zooplankton organisms also explain why the NBSSz slope was significantly steeper in the Estuary and the Bay than on the Shelf.

However, this pattern was only found for $NBSS_Z$. When including particles in the analysis ($NBSS_P$ and $NBSS_N$), all differences in slope between areas became non-significant ($p > 0.05$, Nemenyi post-hoc test). This marked change in comparing slopes was one of the main differences between NBSS analyses with and without particles.

In spite of parallel slopes, intercepts were significantly higher in the Estuary than in the other two areas, after including particles in the analysis ($NBSS_P$ and $NBSS_N$). This is due to the extremely high total volume of biogenic particles in the estuary, and the generally parallel shapes of the NBSS for biogenic particles in the three areas (green lines in Fig. 3).

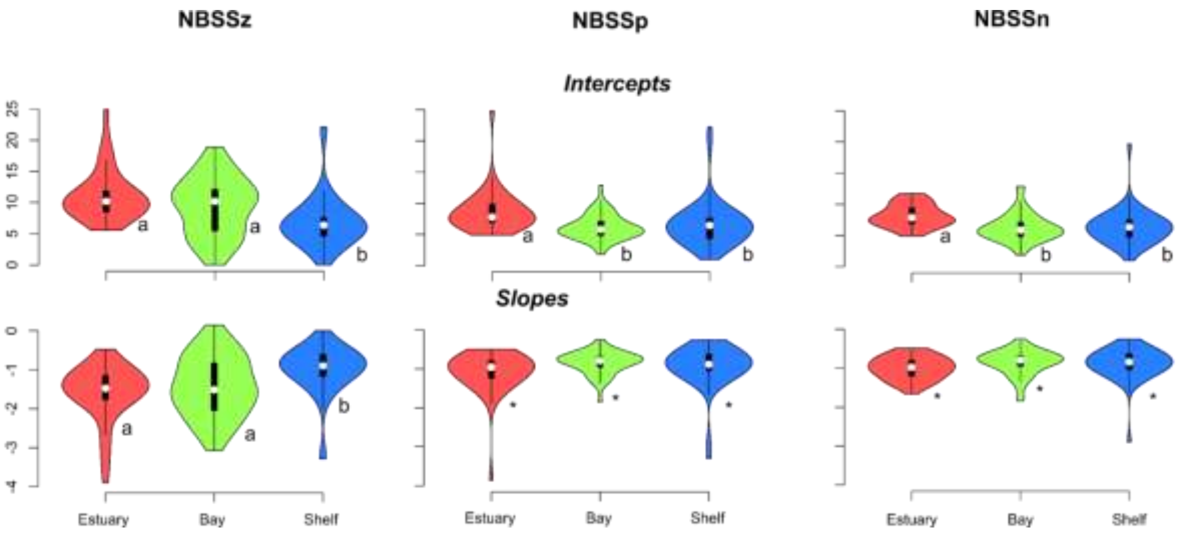


Fig.5. Summary (violin plots, with median, range, interquartile ranges and kernel density distribution) of Normalized Biomass Size Spectra (NBSS) parameters (intercepts and slopes) between three areas (Estuary, Bay, Shelf). NBSSz: zooplankton only; NBSSp: zooplankton and particles, including microplastics; NBSSn: zooplankton and particles, without microplastics. letters ("a" and "b"): results of Nemenyi pairwise post-hoc tests. *: not significant ($p > 0.05$).

7.3.4 The effect of including particles into NBSS: comparing $NBSS_Z$, $NBSS_P$, and $NBSS_N$

The shape of the spectra changes markedly when inserting particles into the traditional zooplankton-only NBSS analysis, except for the Shelf area (Figure 6, Table 2). In the Estuary and in the Bay, slopes of NBSSz (zooplankton only) were significantly steeper (Table 2) and intercepts were significantly higher than for NBSS with particles (NBSSp and NBSSn). Clearly, the inclusion of particles into the spectrum turned the spectrum flatter, due to the effect of large-sized particles (in relation to the zooplankton).

However, there were no differences, in slopes and intercepts, between complete NBSS with zooplankton, particles and microplastics (NBSSp) and NBSS without microplastics (NBSSn), in any of the sampling areas. Thus, inserting or deleting microplastics from the dataset had no significant influence on the structure of the NBSS linear regression models, probably because the overall variability in NBSS was very high, as compared to the relatively low contribution of quantifiable microplastics to the overall particle + zooplankton volumes (Figures 4 and 6). On the Shelf, there were no differences between $NBSS_Z$, $NBSS_P$, and NBSSN, which may be due to the relatively low numbers of particles, or due to the similar slopes of the NBSS.

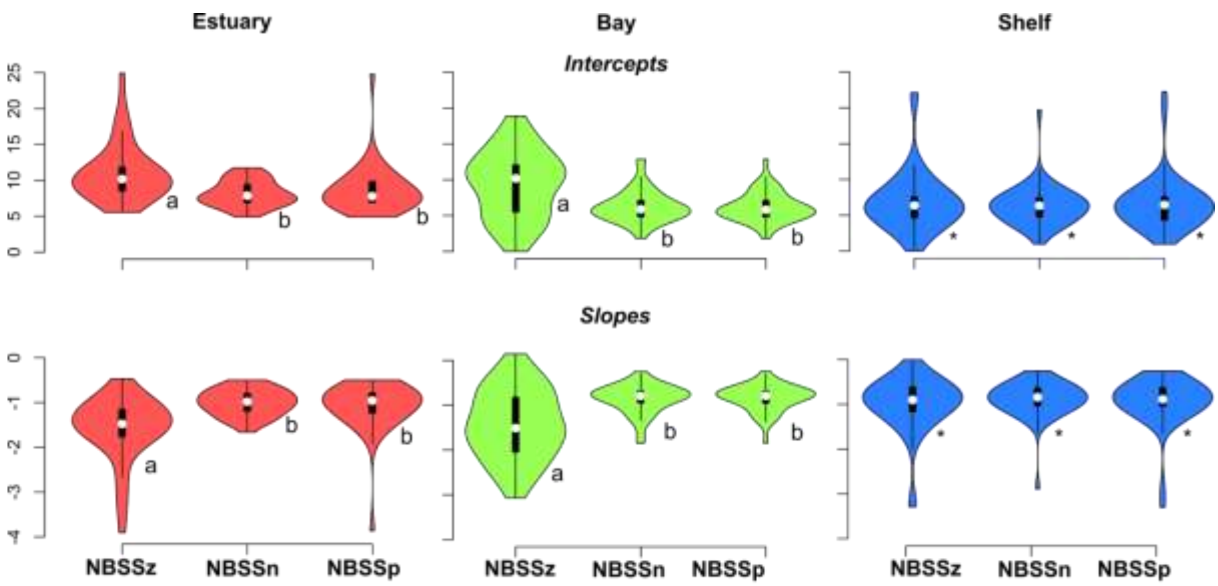


Fig.6. Summary (violin plots, with median, range, interquartile ranges and kernel density distribution) of Normalized Biomass Size Spectra (NBSS) parameters (intercepts and slopes), comparing NBBS of zooplankton only (NBSSz), zooplankton and particles, without microplastics (NBSSn), and zooplankton and particles, including microplastics (NBSSp). letters ("a" and "b"): results of Nemenyi pairwise post-hoc tests. *: not significant ($p > 0.05$).

Table 1. Results of the one-way Permanova and Nemenyi post-hoc pairwise tests for comparing three sampling areas (Estuary, Bay, Shelf). NBSS parameters (slopes and intercepts) of zooplankton only (NBSSz), zooplankton and particles, including microplastics (NBSSp), and zooplankton and particles, without microplastics (NBSSn). The *p*-values are given only when significant ($p < 0.05$). n.s.: not significant. N = 112 samples.

	NBSSz		**NBSSp**		**NBSSn**	
	Slopes	Intercepts	Slopes	Intercepts	Slopes	Intercepts
All Areas (Permanova)	**0.0005**	**0.0001**	n.s.	**0.001**	n.s.	**0.0004**
Estuary *vs* Shelf (Nemenyi post-hoc test)	**< 0.00001 (less steep on the Shelf)**	**0.00001 (higher in the Estuary)**	n.s.	**0.0001 (higher in the Estuary)**	n.s.	**0.0002 (higher in the Estuary)**
Bay *vs* Shelf (Nemenyi post-hoc test)	**0.0025 (less steep on the Shelf)**	**0.0045 (higher in the Bay)**	n.s.	n.s.	n.s.	n.s.
Estuary *vs* Bay (Nemenyi post-hoc test)	n.s.	n.s.	n.s.	**< 0.0001 (higher in the Estuary)**	n.s.	**< 0.0001 (higher in the Estuary)**

Table 2. Results of the one-way Permanova and Nemenyi post-hoc pairwise tests for comparing NBSS parameters (slopes and intercepts) for zooplankton only (NBSSz), zooplankton and particles, including microplastics (NBSSp), and zooplankton and particles, without microplastics (NBSSn). "n.s.": not significant. The *p*-values are given in the table only when significant (p < 0.05). N = 112 samples.

	Estuary		**Bay**		**Shelf**	
	Slopes	Intercepts	Slopes	Intercepts	Slopes	Intercepts
All Areas (Permanova)	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0001**	n.s.	n.s.
NBSSz *vs* NBSSp (Nemenyi post-hoc test)	**0.00001 (steeper for NBSSz)**	**0.001 (lower for NBSSz)**	**0.001 (steeper for NBSSz)**	**0.006 (lower for NBSSz)**	n.s.	n.s.
NBSSz *vs* NBSSn (Nemenyi post-hoc test)	< **0.00001 (steeper for NBSSz)**	**0.0005 for (lower for NBSSz)**	**0.0018 (steeper for NBSSz)**	**0.009 (lower for NBSSz)**	n.s.	n.s.
NBSSp *vs* NBSSn (Nemenyi post-hoc test)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

7.4 Discussion

This study provides the first quantification of a complete particle size spectrum, including zooplankton, microplastics, and other suspended particles along a coastal-shelf gradient. We analyzed the contribution of different types of solid suspended particles (> 299 µm esd), from anthropogenic origin or not, as ecosystem components, in the light of NBSS theory, by means of including zooplankton only (NBSSz), zooplankton, particles & microplastics (NBSSp), and zooplankton and particles, excluding microplastics (NBSSn). This approach allowed novel interpretations of the seston size distribution in aquatic ecosystems.

7.4.1 Zooplankton composition, abundance, size and biovolume in estuarine and marine waters

In this study, copepods were the most abundant group and were present in all sampling campaigns and in all areas. Copepods are well known as the dominant group in marine zooplankton, being present in all marine environments and at all depths (Silva et al., 2003; Costa el al., 2008). We also observed a peak in abundance of the phylum Bryozoa (cyphonautes larvae) in August in the Bay, probably because this group of sessile organisms is common in reef ecosystems (Margulis and Schwartz, 1998). A high abundance of decapods was also observed in the estuarine area, and followed the coastal-shelf gradient which could possibly be linked to their life cycle. During their adult or juvenile phase, many decapods inhabit shallow coastal waters, such as estuaries or mangroves, (Schwamborn and Bonecker, 1996; Melo Júnior et al., 2007, Schwamborn et al., 2008). During the larval phase, the spines that exist in some species seem to contribute to the reduction of predation of larvae (Ruppet et al., 2005). The phylum Chaetognatha also showed a high abundance, mainly at shelf stations. This group of gelatinous predators is often abundant in marine waters (Harzsch et al., 2015).

Biovolume was used to assess the distribution patterns of zooplankton, and for the NBSS, thus considering the body size and biovolume (a proxy of biomass) of each organism and not only the contributions in numerical terms (Dai et al., 2016). Our results showed that different taxonomic groups differed in their contribution to biovolume when compared to abundance. For example, the contribution of large-sized taxa, such as decapods and chaetognaths, was much more important in units of volume than abundance. Conversely, small-sized, abundant cyclopoid copepods had only a minor contribution to the total biovolume. This emphasizes those taxa that are less abundant, but of larger body size, can contribute more to the total biovolume and biomass than abundant small-sized organisms.

However, for the secondary production, small-sized organisms are often especially important due to their fast growth and reproduction rates (Hirst and Lampitt, 1998; Hirst and McKinnon, 2001; Hirst and Bunker, 2003; Lopes, 2007).

7.4.2 Gradients in zooplankton size spectra slopes from estuarine to shelf waters

This study constitutes the first comparison of zooplankton size spectra between tropical estuarine, coastal and shelf areas. The highly productive estuarine ecosystem had the highest intercepts and the steepest zooplankton NBSS (NBSSz) slope, while the oligotrophic (“blue water”) Shelf had the shallowest slopes, close to -1 (i.e., the theoretical mean, that indicates a stable marine ecosystem). Analyzing zooplankton only (NBSSz), the slopes were much steeper than −1 in the Estuary (-1.6) and in the Bay (-1.4), probably due to the importance of small-sized copepods in these productive nearshore areas. The high abundance of small-sized copepods (e.g., cyclopoids *Oithona* spp. and the harpacticoid *Euterpina acutifrons*) has already been well documented in tropical estuarine and nearshore areas in northeastern Brazil (Nascimento-Vieira et al., 2010; Melo et al., 2010, Neumann Leitão et al., 2019b). On the other hand, offshore ecosystems off northeastern Brazil have been characterized by the dominance of large-sized copepods, such as *Undinula vulgaris* (Neumann Leitão et al., 2019b).

These findings suggest that most of the zooplankton communities were characterized by a bottom-up marine ecosystem according to a flattening slopes over a coastal-oceanic gradient due to higher contribution of larger organisms in oceanic areas and the higher small-sized zooplankton concentration near the coast as a consequence of nutrient inputs that boost primary production in coastal environments (Platt, 1985; Marcolin et al., 2013; Sato et al., 2015; Figueiredo et al., 2020).

Both NBSS slopes and intercepts differed between areas, which indicates that different processes may be driving spatial changes in the size structure of zooplankton, microplastics and other particles. The NBSS slope is an index that has been used to evaluate productivity, transfer efficiency, and predation in marine ecosystems (Platt, 1985; Zhou et al. 2009; Marcolin et al. 2013; Sato et al. 2015). Steeper NBSS slopes have been typically associated with high productivity and low trophic transfer efficiency in food webs. On the other hand, shallower NBSS slopes have been associated with low productivity and better trophic transfer efficiency (Sprules and Munawar 1986; Gaedke et al., 2004; Zhou et al. 2009).

7.4.3 The shape of the complete size spectrum (including particles)

This study showed that the inclusion of particles in the size spectra analysis turned the slope less steep in the Estuary and in the Bay. In a simplistic trophic interpretation, the inclusion of particles allowed a higher trophic efficiency in the ecosystem. Some studies demonstrate that several plankton organisms, such as copepods and decapods, feed on organic detritus available in the environment (Schwamborn et al., 2006; Neumann-Leitao et al., 2019). Microplastics, including nylon and PP & PE, were distributed over the same size range as the typical mesozooplankton, from estuarine through shelf waters, during both dry and rainy seasons.

Microplastics were relevant within specific size ranges, especially for larger particles. In the Rio Formoso Estuary large-sized microplastics (> 6.5 $\log_{10}$ mm^3) had a high total volume in both seasons, being similarly important (in terms of volume) as zooplankton, within their size range. Large-sized PP&PPE beads (mean particle volume: approx. 7 to 8 $\log_{10}$ mm^3) were the dominating particles in the water column, in their size range, in the Bay (in both seasons) and long nylon fibres (mean particle volume: approx. 7 to 8 $\log_{10}$ mm^3) on the Shelf, in the rainy season.

The high relative biovolume of large-sized microplastics is unexpected and has very implications for marine ecosystems. It is well known that planktivorous fish generally concentrate on the largest available prey items (Lazzaro, 1987). This aspect turns the impact of large-sized particles on pelagic food webs in all the three samping areas especially worrisome. Our results confirm a recent study (Lins-Silva et al., 2021) that was based on bulk (non-size-stratified) analysis, adding new information regarding the size fractions that are most impacted by microplastics, and regarding the shape of the size spectrum of microplastics and other particles.

However, analyzing NBSS slopes and intercepts with or without microplastics particles showed that adding or removing microplastics had no significant effect on NBSS slopes and intercepts. This could probably be explained by low the abundance and volumes of microplastics, compared to the sum of biogenic particles + zooplankton and to the overall very high variability in NBSS, or by a similarity of size distributions of microplastics and other particles in the water column. Further studies and analyses are necessary to investigate these aspects in detail. Also, the identification of microplastics with ZooScan + FTIR, the non-destructive approach used in this study, may not be sufficient to classify and quantify some categories, especially the "globose opaque particles", which may also contain microplastics. The only way to circumvent this possible shortcoming would be to chemically remove all biological components in the sample (e.g., by acids, bleaches, enzymes and detergents), ultimately destroying all plankton samples. Several previous studies have attempted to describe the size spectrum of microplastics, based on chemically cleaned samples (e.g., Goldstein et al., 2013, Kedzierski et al., 2019 Kooi & Koelmans, 2019), obviously without a comparison with the size spectrum of zooplankton (biogenic detritus is also completely ignored). Our pioneering study is the first to apply NBSS, based on a non-destructive ZooScan + FTIR approach, to assemble a complete particle size spectrum, including zooplankton, microplastics, and other suspended particles into the analysis. Furthermore, this study constitutes the first description of the size spectrum of biogenic detritus in estuarine, coastal and shelf areas.

Also, it is important to note that the present study does not pretend to perfectly quantify the volume spectrum of all particles in the water column (i.e., total seston, including fragile marine aggregates), since we only analyzed robust particles that can be sampled by common plankton nets. The abundance of biogenic particles may be even higher than suggested by our results, when considering fragile structures ("marine snow") that are not well quantified with plankton nets, but appear as the dominant large-sized POM components when using *in situ* optical methods, such as VPR, LOPC, and UVP (Ashjian et al. 2001, Broughton & Lough, 2006; Herman & Harvey 2006; Forest et al., 2012). Although marine aggregates were actually very frequent and abundant in our samples, indicating that they are in fact reasonably well sampled (e.g., they were the most important particles on the Shelf in units of abundance and volume), obviously their real abundance in the water column is much higher than in any data derived from plankton nets, considering their fragile nature. A “perfect” quantification of the seston is actually not possible, since each method has its selectivity and its specific limitations.

The same also applies to zooplankton (i.e., underestimation of abundance and biomass due to gear avoidance of vagile taxa and destruction of fragile gelatinous species), and is a well known limitation of studies based on plankton nets (Harris et al, 2000). Yet, towing plankton nets is still the most practical and cost-effective way to investigate pelagic ecosystems, and is the base of vast and precious archives of past and ongoing sampling and monitoring programs worldwide, that have not yet been analyzed for their complete size spectrum, considering plankton and particles.

Clearly, more studies are necessary to investigate the size spectra of microplastics in coastal ecosystems. The present study constitutes a first, pioneering effort for a synthetic analysis of zooplankton, microplastics and other particles, inaugurating a new line of size-spectra-based research in marine ecosystems.

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8 CONCLUSÕES GERAIS

Este estudo trouxe novos conhecimentos sobre a composição, concentração (contagens m^{-3}), volume (mm^3 m^{-3}) e o espectro de tamanhos do zooplâncton e das partículas (biológicas, microplásticos e outros) suspensas na coluna de água em três ambientes costeiros tropicais do Nordeste brasileiro, utilizando análise de imagens e espectroscopia FTIR, permitindo o entendimento da estrutura e organização das comunidades zooplanctônicas bem como a tipologia e distribuição das partículas e em quais áreas sua presença pode ser mais deletéria.

Primeira comparação de abundância e volume: partículas vs zooplâncton (capítulo 1)

No primeiro capítulo, foi quantificada, pela primeira vez, a contribuição relativa de partículas não-organísmicas para amostras coletadas com redes de plâncton. Esta abordagem inovadora permitiu avaliar a contribuição relativa das meso-partículas para a biomassa sestônica e comparar medições de peso úmido e análise de imagem usando um equipamento ZooScan permitiu novas interpretações na composição do séston em ecossistemas estuarinos e marinhos. O ambiente estuarino teve os maiores valores de biomassa úmida, abundância e volume de partículas quando comparado com a área marinha, mas ainda muito importante. As relações lineares significativas e positivas encontradas entre os dados de biomassa sestônica (obtidas através da metodologia do peso úmido) e volume (obtidos através das análises de imagem) podem ser usados para estimar a biomassa úmida, entretanto, em ambientes marinhos tropicais esses valores podem ser superestimados por causa da alta contribuição das partículas em amostras planctônicas coletadas com rede comum de plâncton.

Primeira avaliação da abundância e do volume: microplásticos vs outras partículas vs zooplâncton (capítulo 2)

No segundo capítulo, partículas biogênicas e microplásticos foram identificados e quantificados, pela primeira vez, por análise de imagem (ZooScan) e por espectroscopia em infravermelho (FTIR). Concentrações mais altas de PP (polipropileno), PE (polietileno) e microplásticos totais (PP + PE + náilon) no estuário indicam um gradiente de diminuição de fontes terrestres em direção ao oceano. As maiores concentrações de fibras de náilon foram encontradas na área da plataforma. Também propomos um novo índice para avaliar a contaminação por microplásticos através da RMC (Concentração Relativa de Microplásticos, em %) considerando que os microplásticos possuem efeito mais deletério quando há menor disponibilidade de alimentos no ambiente. O RMC indicou que a Baía foi o ecossistema mais severamente impactado. A análise da RMC forneceu uma nova perspectiva sobre o impacto dos microplásticos nas teias alimentares costeiras tropicais.

Primeira avaliação conjunta dos espectros de tamanhos do zooplâncton, dos microplásticos e das demais partículas em suspensão (capítulo 3)

Por fim, o terceiro capítulo apresentou, pela primeira vez, a distribuição conjunta de tamanhos e biovolumes do zooplâncton e das partículas biogênicas e microplásticos. O terceiro capítulo forneceu a primeira quantificação de espectro de tamanhos completo, incluindo zooplâncton, microplásticos e outras partículas suspensas na coluna d'água, ao longo de um gradiente do estuário do Rio Formoso até a parte central da plataforma continental ao largo de Tamandaré, Pernambuco (Brasil).

Analisamos a contribuição de diferentes tipos de partículas sólidas em suspensão (> 299 μm esd), de origem antropogênica ou não, como componentes do ecossistema, à luz da teoria do NBSS, por meio da inclusão apenas do zooplâncton (NBSSz), zooplâncton, partículas e microplásticos (NBSSp) e zooplâncton e partículas, excluindo microplásticos (NBSSn). Esta abordagem permitiu novas interpretações da distribuição do tamanho do seston em ecossistemas aquáticos.

As inclinações (slope) e o intercepto do NBSS diferiam entre as áreas, o que indica que diferentes processos podem estar causando mudanças espaciais na estrutura de tamanho do zooplâncton, microplásticos e outras partículas. A inclinação do NBSS é um índice que tem sido usado para avaliar a produtividade, eficiência de transferência e predação em ecossistemas NBSS mais íngremes têm sido tipicamente associados a alta produtividade e baixa eficiência de transferência trófica em cadeias alimentares. Por outro lado, NBSS mais achatados foram associadas a baixa produtividade e melhor eficiência de transferência trófica. Em nosso estudo, o Estuário teve os maiores interceptos e a Plataforma Continental teve os NBSS mais achatados com slope perto de -1 (ou seja, a perto da média teórica, que indica um ecossistema marinho estável). Analisando apenas o zooplâncton (NBSSz), os slopes eram muito mais íngremes do que -1 no Estuário e na Baía, provavelmente devido à importância dos copépodes de pequeno porte em ambas as áreas. Por outro lado, os ecossistemas de plataforma do nordeste do Brasil têm se caracterizado pela presença de copépodos de grande porte. Esses achados sugerem que a maioria das comunidades zooplanctônicas foram caracterizadas por um ecossistema marinho de baixo para cima de acordo com encostas achatadas sobre um gradiente costeiro-oceânico devido à maior contribuição de organismos maiores em áreas oceânicas e à maior concentração de zooplâncton de pequeno porte perto da costa como consequência de entradas de nutrientes que aumentam a produção primária em ambientes costeiros.

Comparando os espectros com zooplâncton e partículas, incluindo microplásticos (NBSSp) e zooplâncton + partículas, excluindo microplásticos (NBSSn), foi possível verificar que a inclusão de partículas na análise de NBSS deixa o slope menos inclinado para todas as áreas de estudo, a inclusão de partículas permitiu uma melhor eficiência trófica no ecossistema. Alguns estudos demonstram que vários organismos do plâncton, como copépodes e decápodes, se alimentam de detritos orgânicos disponíveis no ambiente. Microplásticos, incluindo náilon e PP & PE, foram distribuídos na mesma faixa de tamanho que o mesozooplâncton típico, desde o estuário até as águas da plataforma, durante as estações seca e chuvosa.

A análise NBSS neste estudo revelou que os microplásticos eram extremamente relevantes dentro de faixas de tamanho específicas, especialmente para partículas maiores. No Estuário do Rio Formoso, os microplásticos de grande porte (> 6,5 log10 mm3) tiveram um volume total extremamente alto em ambas as estações, sendo igualmente importantes (em termos de volume) como o zooplâncton, dentro de sua faixa de tamanho. Partículas de PP e PPE de grande porte (volume médio de partícula: aproximadamente 7 a 8 log10 µm3) foram as partículas dominantes na coluna de água, em sua faixa de tamanho, na Baía (em ambas as estações) e fibras de náilon longas (volume médio de partícula: aprox. 7 a 8 log10 µm3) na Plataforma Continental, na estação chuvosa. O alto biovolume relativo de microplásticos de grande porte é inesperado e tem implicações muito sérias para os ecossistemas marinhos. É bem sabido que os peixes planctívoros geralmente concentram seus esforços nas maiores presas disponíveis (Lazzaro, 1987). Este aspecto torna o impacto de partículas de grande porte nas teias alimentares pelágicas em todas as três áreas de amostragem especialmente preocupante.

Os resultados de NBSS confirmam os resultados do capítulo 2 (análises não estratificadas por tamanhos), adicionando novas informações sobre as frações de tamanho que são mais impactadas pelos microplásticos e sobre a forma do espectro de tamanho de microplásticos e outras partículas. No entanto, a análise de inclinações e interceptos de NBSS com ou sem partículas de microplásticos mostrou que a adição ou remoção de microplásticos não teve efeito significativo nas inclinações e interceptos do NBSS. Isso poderia ser explicado pela baixa abundância e volume de microplásticos, em comparação com a soma de partículas biogênicas + zooplâncton, ou por uma distribuição de tamanho semelhante de microplásticos e outras partículas na coluna de água. Claramente, mais estudos são necessários para investigar os espectros de tamanho de microplásticos em ecossistemas costeiros. O presente estudo constitui um esforço pioneiro para uma análise sintética de zooplâncton, microplásticos e outras partículas, inaugurando uma nova linha de pesquisa baseada em espectros de tamanho para melhor compreender e criar novos modelos da poluição por plásticos em ecossistemas marinhos.

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